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Use of VEGF splicing-sensitive fluorescent reporters to screen for anti-angiogenic molecules

USE OF VEGF SPLICING-SENSITIVE FLUORESCENT REPORTERS TO SCREEN FOR ANTI-ANGIOGENIC MOLECULES

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ABSTRACT

Alternative splicing of the vascular endothelial growth factor A (*VEGF-A*) terminal exon generates two protein families with differing functions. Pro-angiogenic $VEGF_{xxx}$ isoforms are produced via selection of the proximal 3' splice site of the terminal exon. Use of an alternative distal splice site creates the anti-angiogenic $VEGF_{xxx}b$ proteins.

A bichromatic splicing-sensitive reporter was designed to mimic *VEGF* alternative splicing and used as a molecular tool to further investigate this alternative splicing event. The terminal exon and preceding intron of *VEGF* were inserted into a minigene construct followed by the coding sequences for two fluorescent proteins. A different fluorescent protein is expressed depending on which 3' splice site of the exon is used during splicing. The fluorescent output can be used to follow splicing decisions *in vitro* and *in vivo*.

Following successful reporter validation in different cell lines and altering splicing using known modulators, small pilot screens were undertaken to search for novel regulators of the splicing decision that creates pro-/anti-angiogenic VEGF isoforms. A larger screen was performed using a library of 1280 small molecules (LOPAC), all compounds of the library were pharmacologically active and have known biological targets. Alterations to reporter splicing were measured using a fluorescent plate reader to detect RFP and GFP expression. Compounds of interest were further validated using flow cytometry and assessed for effect on endogenous *VEGF* alternative splicing. *In vitro* angiogenesis assays were used to demonstrate anti-angiogenic effect. Anti-angiogenic activity and the effect on tumour growth were investigated in several *in vivo* models.

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Firstly, and most importantly, thank you to my fantastic supervisor, Seb, for his constant support, guidance and encouragement during my project. A huge thank you also has to go all my colleagues at UOB: thank you to everyone at the MVRL for guiding through my first steps in the lab and welcoming me to Bristol so warmly; and to new colleagues and great friends from the Renal group, I miss you all already!

I would also like to say thanks to my parents, sisters and wonderful friends for listening to me babble on about the lab and my thesis- even if they didn't have a clue what I was talking about most of the time.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .... DATE: 1/05/16

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ABBREVIATIONS

3’ UTR	3’ untranslated region
5’ UTR	5’ untranslated region
56/1	anti-$VEGF_{xxx}b$
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AF	auxiliary factor
AIDS	acquired immunodeficiency syndrome
Akt	v-akt murine thymoma viral oncogene homolog 1
Ang1	angiopoietin 1
Ang2	angiopoietin 2
ANOVA	analysis of variance
AS	alternative splicing
bFGF	basic fibroblast growth factor
CALCA	calcitonin-related polypeptide α
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
Clk	CDC-like kinase
CMV	cytomegalovirus
Ct	cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DLL4	delta like ligand 4
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyll sulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRED	discosoma red fluorescent protein
DSS	distal splice site
DZnep	3-deazaneplanocin A
ECL	enhanced chemiluminescence
ECM	extracellular matrix

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EMT	epithelial mesenchymal transition
ERK	extracellular regulated kinase, mitogen activated protein kinase
ESE	exonic splicing enhancer
ESRP	epithelial splicing regulatory protein
ESS	exonic splicing silencer
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGFRII	fibroblast growth factor receptor II
FOX1/2	RNA binding protein, fox-1 homolog 1/2
FRET	Forster resonance energy transfer
FTDP	frontotemporal dementia with parkinsonism
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein coupled receptor
HEK293	human embryonic kidney cell line
HGF	hepatocyte growth factor
HIF1α	hypoxia inducible factor 1 α
HIPK3	homeodomain interacting protein kinase 3
HIV	human immunodeficiency virus
HMVEC	human microvascular endothelial cells
hnRNP	heterogeneous ribonucleoprotein particle
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IGF	insulin-like growth factor
IL	interleukin

ISE	intronic splicing enhancer
ISS	intronic splicing silencer
LOPAC	library of pharmacologically active compounds
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MAPK	mitogen-activated protein kinase
MAPT	microtubule-associated protein tau
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
mRNA	messenger RNA
NHE1	sodium hydrogen exchanger 1
NMD	nonsense mediated decay
NO	nitric oxide
NRP1	neuropilin 1
NSCLC	non-small-cell lung cancer
OS	overall survival
PBS	phosphate-buffered saline
PC3	prostate cancer cell line
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PFA	paraformaldehyde
PFS	progression-free survival
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	protein kinase C
PKM	pyruvate kinase M
PLCγ	phospholipase C γ
PlGF	placental growth factor
PP1	protein phosphatase 1
PRC	polycomb repressive complex
PSS	proximal splice site

PTB	polypyrimidine tract-binding protein
PTC	premature termination codon
PVDF	polyvinylidene fluoride
RCC	renal cell carcinoma
RepSox	2-[3-(6-Methyl-2-pyridinyl)-1H-pyrazol-4-yl]-1,5-naphthyridine
RIPA	radioimmunoprecipitation assay buffer
RNA	ribose nucleic acid
RNA pol II	RNA polymerase II
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SMA	spinal muscular atrophy
SMN2	survival of motor neuron 2
snRNP	small nuclear ribonucleoprotein particle
SR protein	serine/arginine rich protein
SRPK	serine/arginine protein kinase
SRSF	serine/arginine-rich splicing factor
SSFR	splicing-sensitive fluorescent reporter
STAT	signal transducer and activator of transcription
TGFβ	transforming growth factor β
TNFα	tumour necrosis factor α
TRA2	transformer 2
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
$VEGF_{xxx}$	pro-angiogenic VEGF isoforms
$VEGF_{xxx}b$	anti-angiogenic VEGF isoforms
VPF	vascular permeability factor

Chapter 1

Introduction

1.1 ANGIOGENESIS

In species such as nematodes and *Drosophila melanogaster,* oxygen is able to freely diffuse to all cells within the organism. Throughout evolution, in larger organisms, the vasculature has developed as an efficient means to transport oxygen to different tissues (Adams and Alitalo, 2007; Carmeliet, 2005). Vascular development has also provided a way for circulating immune cells to traverse tissues supplying immune protection. Nutrients and growth signals are delivered via blood vessels allowing stimulation of organ morphogenesis (Carmeliet and Jain, 2011). The heart pumps blood into arteries which branch into smaller arterioles which branch further to create capillary beds. Capillaries are the smallest blood vessels and are arranged into an extensive meshwork. It is here that metabolic and gaseous exchange occurs, with blood taken back to the heart through the venous system. This forces almost all cells in a tissue to be within close proximity, around 100µm, of a capillary (Hanahan and Weinberg, 2000).

During embryogenesis, early vascular networks are generated by the differentiation of angioblasts from the mesoderm. Maturation of angioblasts produces endothelial cells, which form a mesh of capillaries. This is the process of vasculogenesis. To increase blood supply into developing tissues, the vasculature is expanded further through angiogenesis: the formation of new capillaries via sprouting from existing vessels (Risau, 1997). Pericytes associate with newly formed endothelial sprouts and act to stabilise the vessel and regulate perfusion between the blood and tissues (Carmeliet and Jain, 2011). There are also several other forms of vessel development aside from angiogenesis. In the process of arteriogenesis, smooth muscle cells also associate with the vessels providing stabilisation. Occasionally, intussusception will occur whereby capillaries will split to form two new daughter vessels.

Functional, regulated angiogenesis is vital for development, homeostasis and wound healing. Abnormal or imbalanced angiogenesis is associated with pathology in various diseases. Ischemia can be caused by lack or by insufficient angiogenesis whereas cancer cells induce angiogenesis to sustain tumour growth.

1.1.1 Mechanisms of angiogenesis

Endothelial cells are arranged in a monolayer, linked to each other by adhesion molecules such as claudins and cadherins. Without angiogenic stimuli, endothelial cells are quiescent with a long half-life. Their proliferation is repressed by autocrine signals and survival factors secreted by associated pericytes including VEGF, FGFs NOTCH ligands and angiopoietins (Carmeliet and Jain, 2011). Angiogenesis is a highly regulated process with many complex signalling pathways required. Different combinations of angiogenic molecules stimulate different cells and stages of vessel formation.

Hypoxia is a key angiogenic stimulus. Angiogenesis is induced in response to hypoxia in order to increase oxygen supply to a tissue. Endothelial cells possess oxygen-sensing mechanisms. The prolyl hydroxylase domain (PHD) proteins act as oxygen sensors. When oxygen levels are adequate, PHDs add hydroxyl groups to hypoxia inducible factor proteins (HIF-1α and HIF-2α). Hydroxylation targets the proteins for degradation via association with Von Hippel-Lindau (VHL) protein, which functions in a ubiquitin ligase complex. Under hypoxic conditions, PHDs do not hydroxylate HIFs, preventing their degradation. HIFs are transcription factors and upregulate the expression of many angiogenic proteins including VEGF, nitric oxide synthase and PDGF (platelet derived growth factor).

When endothelial cells are exposed to an angiogenic signal, (such as VEGF, ANG-2, FGFb) junctions between the monolayer of endothelial cells become loose making the vessel more permeable. Basement membrane proteins are cleaved by matrix metalloproteinases to allow remodelling and associated pericytes move away from the capillary wall. Due to the increase in vessel permeability,

plasma proteins can travel from the blood and begin to form new extracellular matrix as a scaffold for the nascent growing vessel. One endothelial cell, known as the tip cell, is chosen to lead the newly forming vascular sprout. Stalk cells, adjacent to the tip, proliferate to elongate the vessel and create lumen within the sprout. Notch signalling is important in regulating tip and stalk cells interaction. Delta-like-4 ligand (DLL4) is highly expressed in the tip cell. Secreted DLL4 activates Notch receptors on neighbouring stalk cells, this is thought to ensure only one cell is selected to lead the new sprout. Tip cells possess long filopodia which detect angiogenic gradients. When a growing vessel meets a capillary or another growing sprout, endothelial cell-cell junctions are created. The formation of the vascular lumen is facilitated by factors including cadherins, VEGF and hedgehog ligands.

As endothelial cell proliferation and vessel elongation stops, pericytes begin to re-associate, stabilising the new vessel. Pericytes are recruited by release of PDGF-B, Notch ligands, ANG-1 and TGFβ. Inhibitors, including TIMPs, block the action of MMPs allowing basement membrane proteins to accumulate and the vessel to mature.

1.1.2 Angiogenesis in disease

Given that angiogenesis is such a fundamentally important process, it is unsurprising that pathology can arise when vessel growth is dysregulated. Many disorders are associated with excessive or insufficient angiogenesis. Neovascularization within the eye can lead to various eye diseases including diabetic retinopathy and age-related macular degeneration (AMD). Retinopathy and wet AMD are caused by irregular growth of vessels in the retina and macula, respectively. Both produce intraocular swelling and bleeding which can lead to permanent blindness (Witmer et al., 2003). Abnormal angiogenesis is also implicated in several disorders associated with the reproductive system conditions. Hyper-vascularisation and increased expression of pro-angiogenic factors is found in polycystic ovary syndrome and endometriosis (Reynolds et al., 2002). In contrast, several other pathologies are related to insufficient

vascularisation. Ischemia of the heart and limbs, stroke and hypertension are both associated with inadequate vessel development. Malformed vessels are also observed in pre-eclampsia, psoriasis and various other conditions (Carmeliet and Jain, 2000).

1.1.3 Tumour angiogenesis

The relationship between sustained tumour growth and an increase in vascularisation has been described since the beginning of the 20th century. In 1927, Lewis described the vascular structures within rat tumours. It was observed that the vascular architecture differs between tumours and proposed that the tumour environment has a pronounced effect on vascular morphology (Lewis, 1927). The use of chamber assays enabled tumour angiogenesis to be clearly observed *in vivo* and was also used to show the association between tumours and vessel growth. It was observed that transplanted cancer cells, but not normal cells, could induce neovascularization. It was hypothesised that tumours acquire the capability to induce angiogenesis and that this is a key step of tumourigenesis (Algire and Chalkley, 1945; Ide et al., 1939). In 1971, Judah Folkman proposed that tumour growth was dependent on angiogenic vessel growth and endothelial cell proliferation could be stimulated by a diffusible factor produced by cancer cells. This factor enables endothelial cells to move from their normal, quiescent phenotype to highly proliferative cells that form new sprouting blood vessels. Tumour growth stalls without neovascularisation, therefore, solid tumours larger than 1-2mm are always associated with blood vessels predominantly via neovascularisation. Folkman coined the term “anti-angiogenesis” as a method of cancer therapy and hypothesised that tumours may be most vulnerable to treatment in the short period before they induce neovascularisation (Folkman, 1971). Sustained angiogenesis was included as one of the defining features common to all solid cancers in the seminal paper, The Hallmarks of Cancer (Hanahan and Weinberg, 2000). It is now clearly established that angiogenesis is a rate-limiting step during tumourigenesis and metastasis.

The stimulation of vasculature expansion by the tumour has been traditionally thought of as being controlled by the 'angiogenic switch'. The switch is controlled by the ratio between positive and negative regulators of angiogenesis. Tumours secrete a variety of factors that simulate angiogenic growth. The VEGF family of proteins, including VEGF-A, VEGF-C, VEGF-D and PlGF, are all expressed by cancer cells. FGF and ANGs are also implicated. VEGF-A is a principle pro-angiogenic molecule during tumourigenesis. The hypoxic microenvironment of a tumour can induce the release of many growth factors from the cancer cells. As cancer cells divide to increase the tumour volume, oxygen availability is reduced. Hypoxia stimulates transcriptional upregulation of pro-angiogenic molecules. The activity of certain oncogenes within cancer cells also contributes to the increased expression of pro-angiogenic factors. Acidity, hypoglycaemia and inflammation also participate in the induction of the angiogenic switch (Bergers and Benjamin, 2003).

Before the induction of neovascularisation, small tumour lesions are avascular, only tumours that induce angiogenesis can continue to expand (Figure 1-1). Angiogenesis may be induced at varying stages during tumour development depending on the cancer type and the surrounding microenvironment. Furthermore, the vessel growth stimulated by tumours is different from angiogenesis that occurs physiologically. Normal sprouted vessels mature quickly and become stable structures. In tumour angiogenesis, the unbalanced expression of pro vs. anti-angiogenic regulators results in structurally abnormal vessels. The shape of induced tumour vessels is disorganised and tortuous. The capillaries within tumours are highly branched with variable diameter of the vessel lumen. The vessels are also more permeable than normal, prone to haemorrhage and have irregular interaction with pericytes. Due to the chaotic organisation of tumour vasculature, the blood flow through the tumour is irregular causing regions of hypoxia and acidity within the tumour. It has been demonstrated that tumour cells can become incorporated into the vessel wall forming a mosaic with endothelial cells (Carmeliet and Jain, 2000).

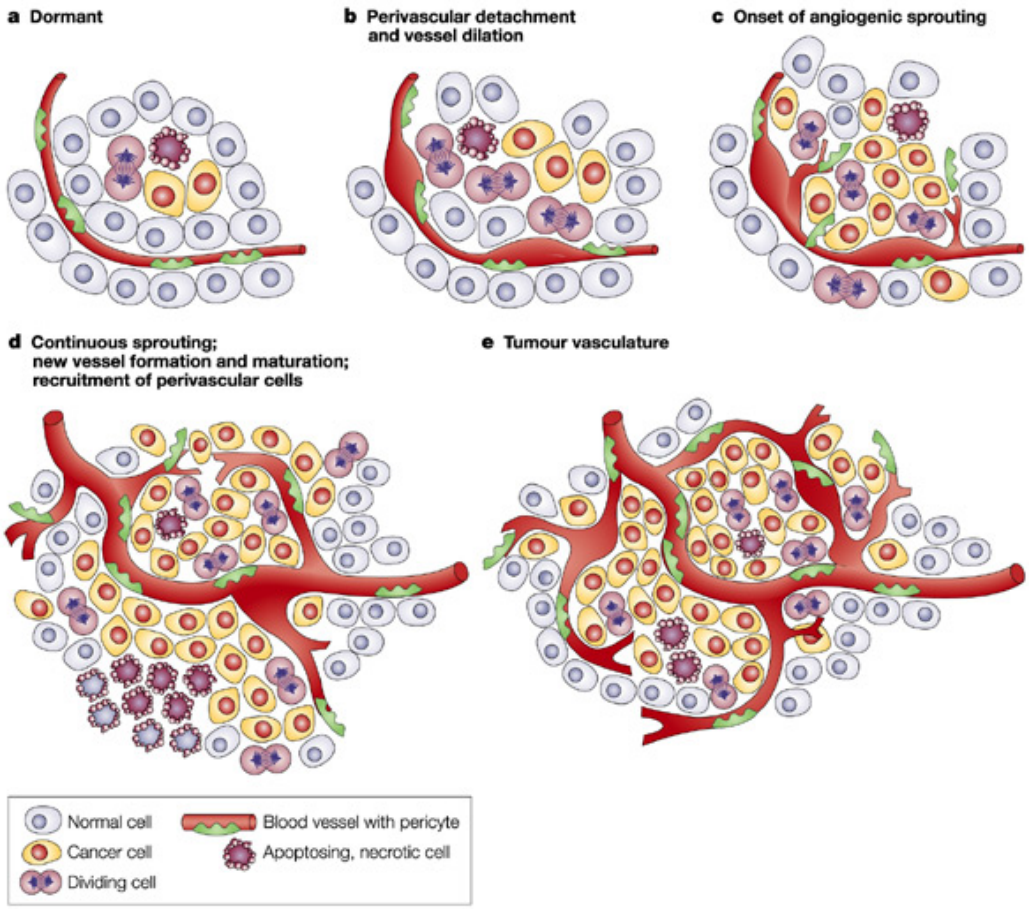


Figure 1-1 Tumour angiogenesis

(A) During the early stages of tumour growth, cancer cells rely on pre-existing blood vessels for nourishment. (B) Hypoxia and factors secreted by the tumour stimulate dilation of vessels and detachment from the basement membrane in the angiogenic switch. (C) Nascent vessels begin to sprout through angiogenesis. (D) Vessel growth continues with the vessels becoming more mature and associated with pericytes. (E) Tumour can rapidly expand with its own vasculature. Image from Bergers and Benjamin, 2003.

1.1.4 Anti-angiogenesis as a therapeutic strategy

Angiogenesis is infrequent in the healthy adult, but a major process in many pathologies. This makes angiogenesis an attractive therapeutic target as it would be expected for side effects to be limited. The growth factors and receptors that mediate angiogenesis have been extensively investigated as drug targets with several drugs approved for clinical use and many more in development. Most of the approved therapies, for cancer and other angiogenic diseases, are anti-VEGF agents.

The humanized monoclonal antibody, bevacizumab, was the first angiogenesis inhibitor to be used clinically in the United States (Ellis, 2005). Bevacizumab binds VEGF blocking its interaction with VEGFRs on the vascular endothelium. Mouse models of angiogenesis were used to show the angiogenic inhibition via VEGF blockade could impede tumour growth (Kim et al., 1993). The first trials compared the effect of chemotherapeutic agents on colorectal cancer when combined with bevacizumab or alone. Patient progression free survival (PFS) and median overall survival (OS) were increased significantly by the use of bevacizumab alongside chemotherapy (Hurwitz et al., 2004). The effectiveness of bevacizumab has varied among different cancers. Neither PFS or OS were changed when used in combination with capecitabine to treat metastatic breast cancer (Miller et al., 2005). Bevacizumab is currently an approved therapy for the treatment of metastatic colorectal cancer, non-squamous non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC) and glioblastoma (Cook and Figg, 2010).

As well as directly targeting VEGF, many attempts have been made to inhibit the kinase activity of VEGF receptors. Sunitinib is a small molecule inhibitor of VEGFR-2 and also has inhibitory action on PDFGR, Flt-3 and other receptor tyrosine kinases. The inhibitor is used for treatment of gastrointestinal tract tumours and renal cell carcinomas (Gan et al., 2009). The Raf inhibitor, sorafenib, has been shown to have anti-angiogenic activity via inhibition of VEGFR-2,

VEGFR-3 and PDGFRβ and provides survival benefit in hepatocellular cancers and RCC (Wilhelm et al., 2008).

1.2 ALTERNATIVE SPLICING

1.2.1 Pre-mRNA splicing

It was in 1958 that Crick first proposed 'the sequence hypothesis'. This theory suggested that the nucleotides of genetic material provide the code that determines the amino acid sequence of proteins. This provides the basis for the 'central dogma of molecular biology'- double stranded DNA is transcribed to a single stranded RNA molecule, which is then translated into protein (Crick, 1958). It was originally believed the mRNA molecules translated by ribosomes were exact copies of DNA sequence. Through their work on viral genes, Roberts and Sharp identified so-called 'split genes' (Chow et al., 1977). They showed that in viruses, some mRNAs were shorter than the DNA they were transcribed from with large gaps, introns, cut out of the mRNA before it is translated. It was quickly shown that most eukaryotic gene have this molecular structure, with coding sequence (exons) interrupted by non-coding sequence (introns). Pre-mRNA is transcribed from a gene, introns removed and exons are re-joined by the process of splicing to create mature mRNA transcripts.

For many years it was widely accepted that one gene holds the information to create one polypeptide chain of amino acids (Beadle and Tatum, 1941). This proved to be an oversimplification. Sequencing of the human genome revealed there to be far fewer genes than expected, especially in comparison to the number of proteins that make up the proteome. The diversity of expressed proteins is increase by various post-translational and post-transcriptional modifications with one of the most important being alternative splicing. Early estimates predicted around 60% of human genes to be alternatively spliced (Modrek and Lee, 2002). More advanced transcriptomics has now revealed 92-94% of genes may undergo alternative splicing (Pan et al., 2008). Comparison of the transcriptomes between species has demonstrated that humans and other primates have a higher frequency of alternative splicing events compared to

other vertebrate species. Differences in alternative splicing patterns perform a key role in creating species diversity. Around 50% of alternative splicing events are conserved between human and chimp. Conservation is further reduced in mice, to less than 30% commonality with humans (Barbosa-Morais et al., 2012).

1.2.2 The spliceosome

The molecular machinery that performs mRNA splicing is the spliceosome, a large complex of protein and RNA molecules. The spliceosome components assemble on an mRNA transcript and catalyse two transesterification reactions to remove the intron and join exons. Splice sites, at the exon-intron boundary, are partially conserved sequences that are recognised by the splicing machinery. 5' splice sites have a conserved GU dinucleotide with the surrounding sequences less conserved. At the end of the intron is a 3' splice site, which has two conserved bases, AG. Around 15-50 bases upstream of the 3' splice site is the branch point, an adenosine that is critical for splicing catalysis is usually found within this motif (Figure 1-2A). The branch point is followed by the polypyrimidine tract, a sequence rich in uracil and cytosines.

The splicing reaction is guided by five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5 and U6, with many other associated proteins. SnRNPs associate with transcripts via RNA-RNA base pairing with short nucleotide motifs. The mechanisms of spliceosome assembly and catalysis were predominantly discovered using yeast as a model organism. During the first step of the splicing reaction, the phosphate group of the guanine nucleotide at the 5' splice site is nucleophilically attacked by the 2' hydroxyl group of the branch point adenosine (Figure 1-2B). The upstream exon is released from the intron, which has formed a lariat loop with the 5' splice site joined to the branch point. In the second transesterification step, the final phosphate of the intron is attacked by the detached exon (Figure 1-2C). The intron lariat is released and the two exons joined (Matera and Wang, 2014).

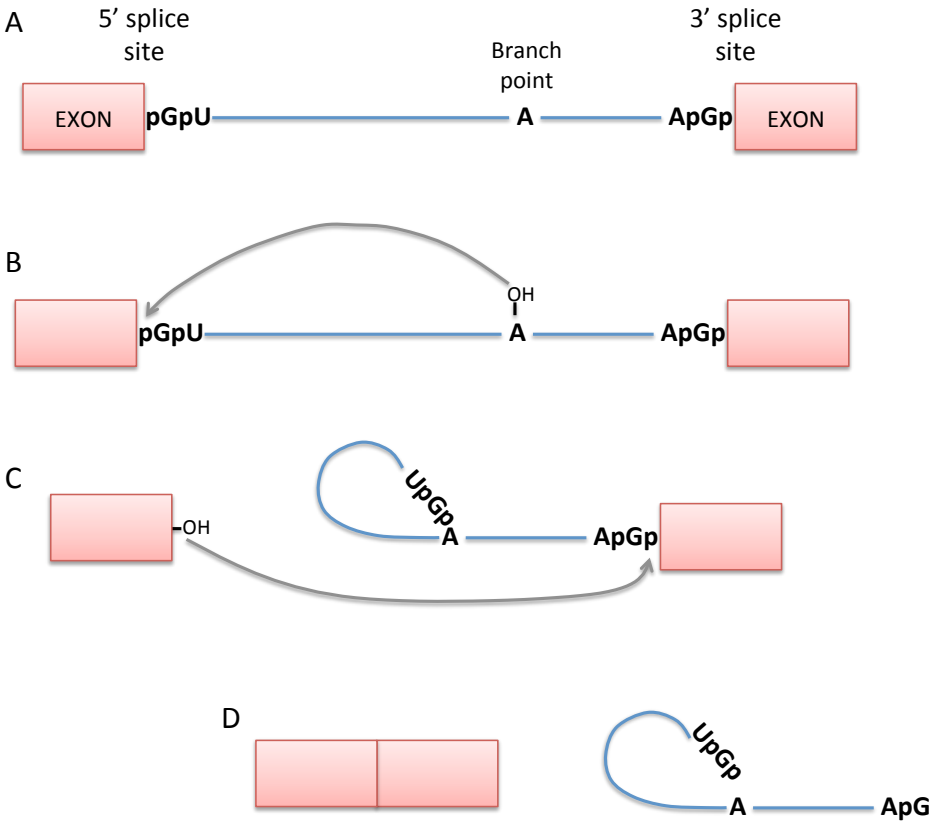


Figure 1-2 The splicing reaction

(A) The 5' and 3' splice sites form the boundaries between intron and exons. (B) The first transesterification step joins the 5' end of the intron to the branch point. (C) In the second step, the 5' exon attacks the 3' splice site. (D) The two exons are joined and the intronic lariat is released. Adapted from Black, 2003.

The spliceosome arranges into several different complexes through the different stages of the splicing reaction (Figure 1-3). Exons are first recognised through splice sites in the process of exon definition. Early spliceosome assembly is centred around exons due to the much larger length of introns. This facilitates splice site pairing to decide which sequence is removed from the transcript (Berget, 1995). The early components of the spliceosome make up complex E. This stage is characterised by U1 snRNP associating with the 5' splice site via base pairing. U2AF proteins 35 and 65 recognises the 3' splice site and polypyrimidine tract. Another spliceosomal protein component, SF1, binds the branch point (Figure 1-3A) (Graveley et al., 2001). E complex formation is ATP-dependent. Rearrangement with SF1 being replaced by U2 snRNP at the branch point forms

complex A, the pre-spliceosome (Figure 1-3B). Interaction between U1 and U2 from the 5’ and 3’ end of the intron makes the switch from exon to intron definition. Complex A brings the splice sites and branch point within close proximity. The pre-assembled tri-snRNP, formed by association of U4, U5 and U6 snRNPs, joins the mRNA molecule creating complex B (Figure 1-3C). The catalytically active complex B* is formed by further rearrangements releasing U1 and U4 from the spliceosome and initiating the first transesterification reaction (Figure 1-3D). The 5’ exon and intron lariat-exon intermediate are bound by U2, U5 and U6 snRNPs as the C complex (Figure 1-3E). The second transesterification, to join the two exons, is carried out by complex C. U2, U5 and U6 are released from the spliced mRNA bound to the intron lariat (Figure 1-3F) (Matera and Wang, 2014; Wahl et al., 2009).

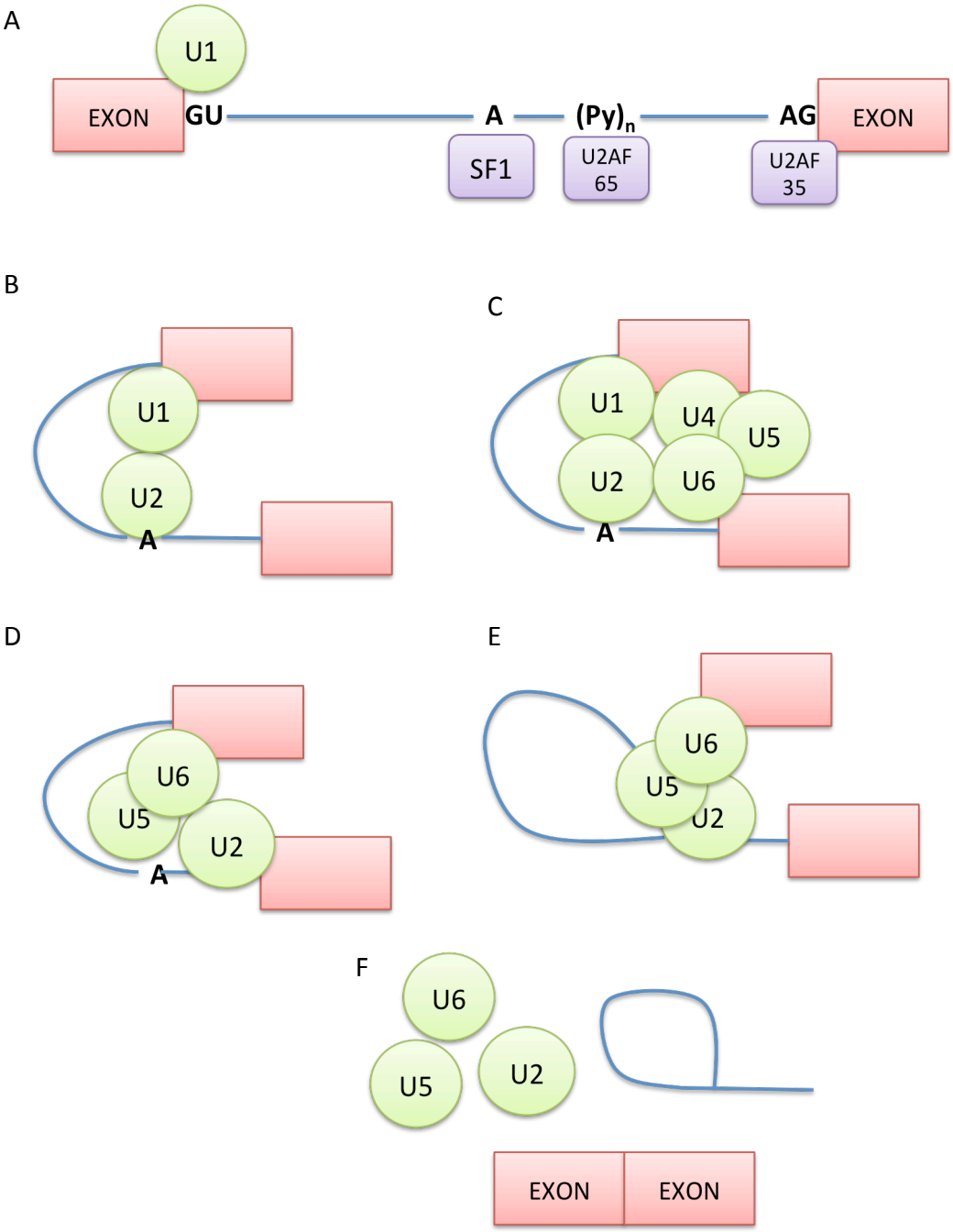


Figure 1-3 Spliceosome assembly and splicing catalysis

(A) E complex is formed by U1 snRNP binding the 5' splice site and other factors associating with the branch point and 3' splice site. (B) U2 snRNP replaces SF1 at the branch point forming A complex. (C) B complex is formed by tri-snRNP joining the spliceosome. (D) U1 and U4 dissociate from the pre-mRNA. Rearrangements make the B complex which catalyses the first transesterification. (E) The C complex catalyses the second reaction. (F) Two exons are joined and intron lariat released. Adapted from Black, 2003.*

1.2.3 Alternative splicing: a mechanism to increase proteome diversity

Splicing is the process of removing intronic sequences and joining exons of mRNA transcripts. The majority of human genes are multi-exon with an average length of an exon between 50 and 250 bases. Introns are far longer, usually thousands of bases. Alternative splicing allows varying combinations of splice sites to be used changing which exons or parts of exons are included in the final transcript. Multiple mature mRNAs can be produced from a single gene leading to different protein isoforms with altered amino acid sequences. This can cause changes to the structure and function of the final protein (Black, 2003).

Splicing mechanisms have been intensively studied in yeast. The splice sites in *Saccharomyces cerevisiae* are highly conserved; this is not the case in higher metazoans with far more variability in splice site sequences. This may be an important feature in creating varied patterns of alternative splicing and produce the need for many regulatory factors to promote or inhibit splice site use. Genome sequencing has shown that humans do not possess many more genes than many lower organisms despite being far more complex species. It is possible that more opportunity for alternative splicing contributes to the increased complexity of higher organisms (Kim et al., 2007).

The type of alternative splicing can be classified into several categories (Figure 1-4). Constitutive exons are always included in the transcripts produced from a particular gene. A cassette exon will be included in the final transcript in some situations and excluded in others. Some genes have mutually exclusive cassette exons, whereby one or the other, but never both exons, are included in the mature mRNA. Some exons have multiple 5' and 3' splice sites, allowing the length of the exon to be changed by alternative splicing. Intron retention is a form of alternative splicing in which the intron remains as part of the final mRNA transcript. Alternative promoters and polyadenylation sites can also be selected changing the 5' and 3' exons of a transcript (Black, 2003).

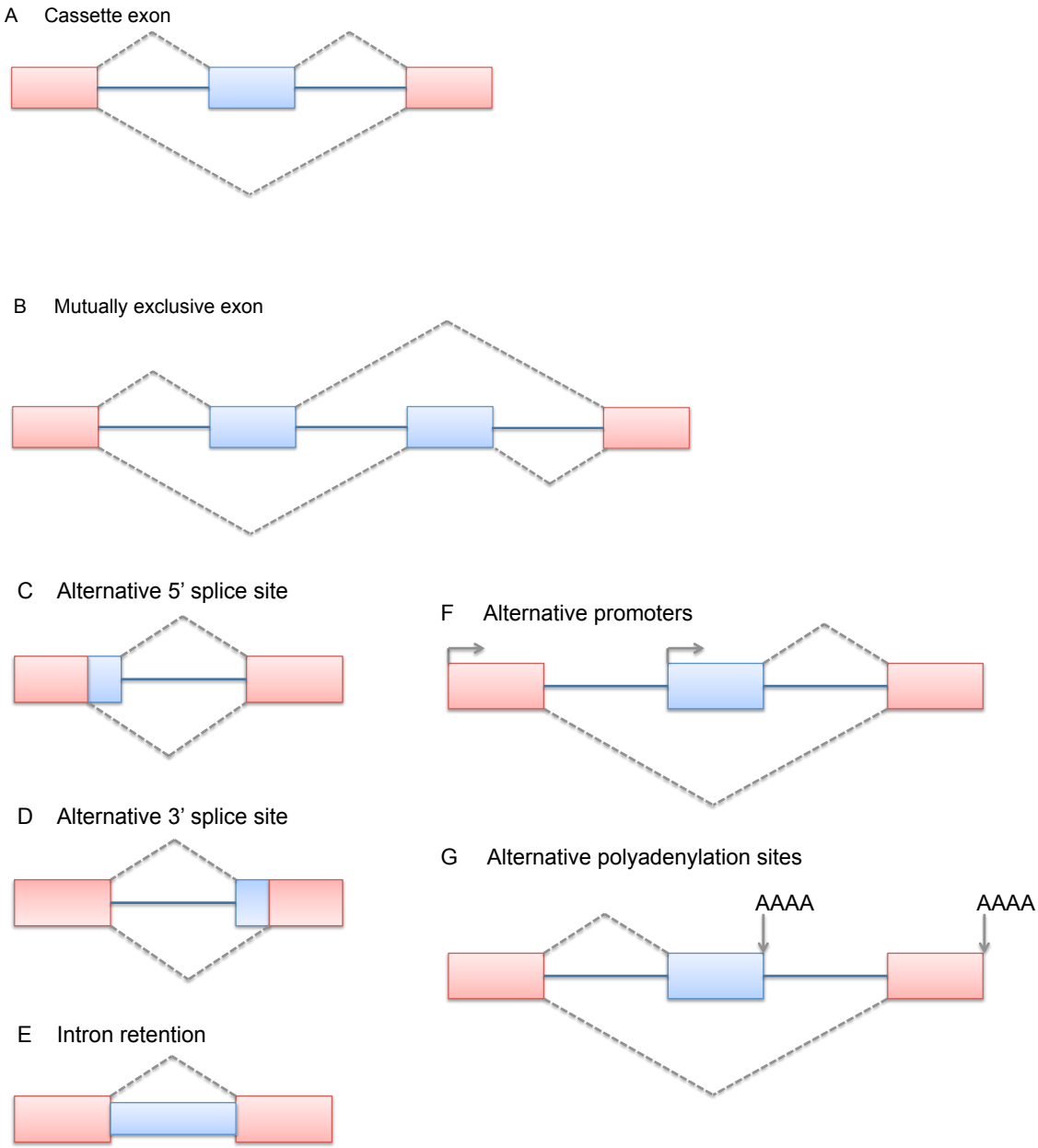


Figure 1-4 Forms of alternative splicing

(A) Skipping or inclusion of an alternatively spliced cassette exon. (B) Mutually exclusive exons are never both included in the same mature mRNA. (C, D) Alternative 5' and 3' splice sites shorten or lengthen an exon. (E) intron retention often produces a truncated protein due to stop codons present in the intronic sequence. (F, G) Use of alternative promoters and polyadenylation sites result in different proteins from the same transcript but they are not purely an alternative splicing mechanism. Adapted from Black, 2003.

1.2.4 Regulation of alternative splicing

Splicing removes introns and joins exons in pre-mRNA transcripts from multiexonic genes. Constitutively spliced exons are efficiently recognised by the splicing machinery and follow the same pattern of splicing in all transcript species from a particular gene. Alternative splicing allows different combinations of exons to be joined resulting in a different protein product. 95% of all split genes are alternatively spliced in humans (Pan et al., 2008). During alternative splicing, pairs of 5' and 3' splice sites are in competition with other potential sites. The 'strength' of a splice site will affect how often an exon is spliced, but not all sequences that are predicted as strong splice sites are well used. There are many 'pseudo-exons' within pre-mRNA, which have exonic characteristics but are not normally recognized by the spliceosome.

The decision of which splice sites are used -determining which exons are included in a mature transcript - involves cis-elements within the RNA sequence and trans-acting protein regulators. Cis-splicing regulatory elements (SREs) are divided into four categories depending on their function and location within pre-mRNA transcripts; exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) (Figure 1-5). Recognition of cis-elements by splicing regulatory proteins is involved in determining if a splice site is used. There are numerous RNA binding proteins that recognise SREs and influence splicing decisions. Cis-elements can also influence splicing through the secondary/tertiary structure of the pre-mRNA molecule (Chen and Manley, 2009).

Enhancer sequences are often bound by members of the serine/arginine (SR)-rich protein family, which can enable splice site recognition in both constitutive and alternative splicing. Many SR proteins also have functional roles in other mRNA related processes such as transcript nuclear export, translation and non-sense mediated decay of mRNAs. There are more than ten members of the SR protein. At their N termini, SR proteins posses at least one RNA recognition motif (RRM) domain allowing them to bind transcripts. SR proteins have many serine

and arginine residues making up the RS domain, which can be phosphorylated to regulate the protein activity (Long and Caceres, 2009). SRs can facilitate the recruitment of the U1 snRNP and U2/U2AF complexes to particular splice sites by association with an exonic enhancer sequence. SR proteins can form part of large enhancing complexes with other splicing promoting factors including transformer 2 (TRA2) and SRm proteins. Splice site recognition and recruitment can also be facilitated by binding to intronic enhancers. SAM68 promotes use of a 3' splice site in CD44 transcripts through recruitment of U2AF (Tisserant and Koenig, 2008). In other cases, U1 snRNP recruitment to weak 5' splice sites is enhanced by the protein T cell restricted intracellular antigen 1 (TIA1) binding to a downstream intronic region (Forch et al., 2002).

Silencer sequences are highly variable. Some are known to be bound by hnRNPs (heterogeneous ribonucleoprotein particle). hnRNPs also contain RRM domains and also KH RNA binding domains. The hnRNP family have various roles in mRNA processing. hnRNPA1 and PTB (polypyrimidine tract binding protein) are known splicing repressors. Splicing regulatory proteins frequently act by affecting early spliceosome formation. Silencer sequences can inhibit splicing by interfering with the binding of snRNPs to nearby splice sites. For example, PTB association with the polypyrimidine tract can prevent U2AF binding required for spliceosome assembly (Sauliere et al., 2006). FOX1 and FOX2 are important splicing factors in muscle and neurons. FOXs prevent spliceosome assembly on *CALCA* transcripts by binding an intronic sequence blocking SF1 association with the branch point. Inhibitory splicing factors also can reduce use of a splice site by preventing the action of positive regulator proteins binding to enhancer sequences. FOX1 and 2 bind a sequence in a *CALCA* exon near an ESE binding site for TRA2, which is used to recruit U2AF (Zhou and Lou, 2008). But some silencer sequences are located hundreds of bases away from enhancers and splice sites; therefore blocking enhancer binding does not explain their silencing function. These sequences may facilitate the formation of loops in the pre-mRNA molecule structure, altering spliceosome formation. Splice sites and ESEs/ISEs can be masked by changes in the secondary structure of pre-mRNA. Ultimately, splicing decisions are

controlled by the combination of activators and repressors. Some splicing regulatory proteins can even inhibit or induce splicing when bound at different positions on the RNA (Ule et al., 2003). For example, some cassette exons can be spliced or silenced by hnRNPL depending on which downstream sequence it associates with. NOVA1 binds an exonic silencer sequence in its own pre-mRNA but promotes inclusion of exons in other genes (Dredge et al., 2005).

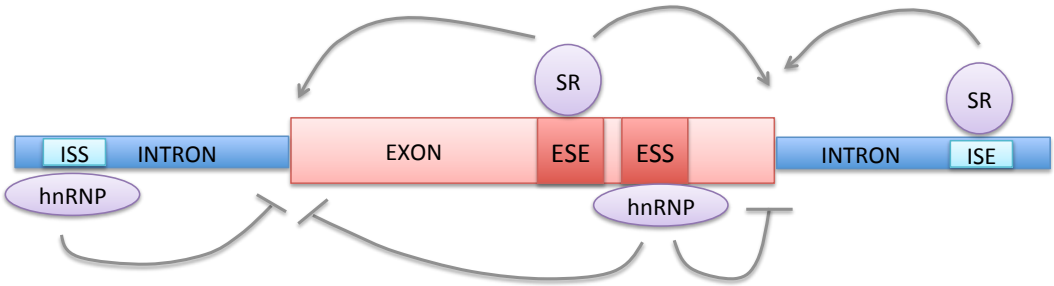


Figure 1-4 Regulation of alternative splicing by SREs and trans-acting factors

Splicing regulatory elements (SREs) are sequences within mRNA transcripts that are bound by splicing regulatory proteins. SREs are classified according to their location and activity: ISS (intronic splicing silencer), ESE (exonic splicing enhancer), ESS (exonic splicing silencer), ISE (intronic splicing enhancer). SR proteins commonly bind enhancers promoting selection of a certain splice site. hnRNPs predominantly act as negative regulators. Adapted from Black, 2003.

Understanding splicing regulation becomes further complex by considering that splicing occurs co-transcriptionally. RNA binding proteins quickly associate with the nascent pre-mRNA transcript as it emerges from RNA polymerase II. Splice factors may interact with RNA polymerase II to enhance or inhibit the use of a particular splice site. There is also evidence indicating the speed of transcription is associated with selection of alternative splice sites. Phosphorylation of RNA polymerase II can alter the rate of pre-mRNA synthesis potentially influencing splicing (Naftelberg et al., 2015).

1.2.5 Cell signalling pathways that regulate alternative splicing

Differential alternative splicing occurs between tissues, often via tissue specific expression of many regulatory proteins. But various stimuli can the alter pattern

of alternative splicing in a cell without changing the expression of splice factors. Increasing evidence indicates that hormones and growth factors along with other stimuli influence pre-mRNA splicing. Like many other cellular processes, signalling cascades can act to transduce signals to the nucleus in order to modify alternative splicing events.

Post-translational modifications of RNA binding proteins can be induced by signals from within and outside the cell. Various signalling molecules, such as JNK, MEK, PKC and ERK, have been shown to mediate pathways that influence splicing decisions (Pelisch et al., 2005; van der Houven van Oordt et al., 2000; Weg-Remers et al., 2001; Xie and Black, 2001). Phosphorylation of splice factors by kinases can change their localisation within the cell and effect their association with other proteins and RNA molecules. For example, stress signals can induce changes to the MEK4/7-MAPK p38 pathway altering the cellular localisation of hnRNP A1 (van der Houven van Oordt et al., 2000).

SRSF protein kinases (SRPKs) are known to be important modulators of alternative splicing via phosphorylation of SR proteins. Cell signalling by growth factors can be linked to changes in splicing patterns via splice factor phosphorylation. For example, epidermal growth factor (EGF) binding to its cell surface receptor initiates a signalling cascade increasing the activity of SRPK1. SRPK1 is known to phosphorylate the SR protein SRSF1 causing changes in the alternative splicing of SRSF1 target mRNAs. EGF signal transduction is thought to be mediated by Akt, which is involved in regulating phosphorylation of various splicing factors (Shultz et al., 2010; White et al., 2010). Interplay between growth factors and SRPKs/SR proteins may provide key regulation of alternative splicing decisions (Zhou et al., 2012).

1.2.6 Splicing in disease and therapy

Given that splicing is a fundamental and widespread step in the process of gene expression, it is unsurprising that disease can arise from abnormal splicing. Mutations within the genome may affect the sequence of consensus splice sites

or regulatory elements. New cryptic splice sites and enhancers/silencers may be created by mutation or current sites and elements may be damaged (Garcia-Blanco et al., 2004). Many mutations that are predicted to be silent as they do not alter the encoded amino acid may disrupt splicing or other RNA processing mechanisms (Lee and Rio, 2015).

Blood disorders, such as β- thalassemia, can cause acute episodes of anaemia. Some β-thalassemia cases are caused by mutation of the β-globin gene creating a cryptic 3' splice site (Busslinger et al., 1981). Another well-described, splicing-related genetic disease is spinal muscular atrophy (SMA). The disease causes muscle atrophy with anterior horn motor neurons becoming degenerated over time. The phenotype ranges from mild to severe. Two paralogous genes, *SMN1* and *SMN2* produce survival of motor neuron proteins. Loss of the SMN1 gene causes SMA but the severity of the disease is dependant on the protein produced from the *SMN2* gene. *SMN2* has a mutation in exon 7 that increases skipping of the exon during splicing creating non-functional protein and a more severe disease phenotype. It is thought that transition of a single nucleotide in exon 7 may create a silencer sequence or disrupt an enhancer (Singh et al., 2004).

Alternative splicing can produce multiple proteins from a single gene. The balance between expression of differentially spliced isoforms is important and defects in isoform ratios can be disease causing. For example, the microtubule-associated protein, Tau, is encoded by the *MAPT* gene. Alternative splicing produces six Tau protein isoforms, which either include or have exon 10 skipped. Exon 10 encodes one of four microtubule repeats. The isoform balance is very important for appropriate neuronal function. Normally in the brain, there is a 1:1 ratio of protein isoforms with exon 10 included (four repeats) or skipped (three repeats). Many mutations effecting Tau function are linked to development of fronto-temporal dementia with Parkinsonism (FTDP-17). Several mutations have been shown to induce or reduce exon 10 inclusion in *MAPT* transcripts altering the isoform ratio leading to neurodegeneration (Spillantini et al., 1998; Spillantini et al., 2000).

Attempts have been made to change alternative splicing patterns as a method to treat disease. Targeted oligonucleotides have been used to initiate or inhibit certain splicing events by base pairing with an RNA sequence and either blocking binding of regulators or recruiting other factors. One of the diseases that has been intensively investigated in terms of using spicing modifiers as therapeutics is Duchenne muscular dystrophy. Mutations in the dystrophin gene can cause frame shifts in the sequence resulting in premature termination during translation. Loss of dystrophin leads to muscle weakness and heart/respiratory problems, and ultimately, premature mortality. In a mouse model of Duchenne muscular dystrophy, mice have a mutation within exon 23 of the gene encoding dystrophin in exon 23. Dystrophin with exon 23 mutation is not functional due to a frame shift. Antisense oligonucleotides were designed to base pair with exon 23 pre-mRNA and cause it to be skipped. The translated protein has partial functionality. Mice treated with the oligomer had enhanced muscle function (Lu et al., 2003). Similarly, oligo nucleotides have also reached human clinical trials. Kinali et al., delivered a dystrophin-targeting oligo nucleotide to patients with Duchenne muscular dystrophy as an intramuscular injection into the extensor digitorum brevis muscle of the foot. The oligo induced skipping of dystrophin exon 51 during splicing and increased to expression of dystrophin in the treated muscle (Kinali et al., 2009). Additional investigations were performed using intravenous infusions of the same oligo nucleotide as a novel treatment. No adverse side effects were observed and dystrophin protiein expression was increased (Cirak et al., 2011). Various clinical trails using different oligonucleotides have also been conducted (Goemans et al., 2013; Goemans et al., 2011).

Changes in the levels of splice factors and associated proteins can also cause wide-ranging defects. Small molecule inhibitors have been used to modify splicing by interfering with upstream proteins involved in regulating splice factor activity using post-transcriptional modification. For example, inhibiting Clk1 kinase can alter splicing events dependent on one of its target proteins, SRSF1 (Muraki et al., 2004).

1.2.7 Cancer and alternative splicing

Cancer cells exhibit radical changes across all regulatory stages of gene expression including chromatin arrangement, transcription, mRNA polyadenylation, alternative splicing, translation or post-translational modifications. Faulty alternative splicing in cancer has wide-ranging effects on cellular processes such as control of the cell cycle, angiogenesis and invasion and metastasis. Cancer cells can alter the splicing regulatory mechanisms to produce protein isoforms that are beneficial for their survival and growth. Aberrant splicing occurs on a large scale in tumours often caused by distorted expression and/or regulation of RNA binding proteins. hnRNP A1 expression is linked to increased cell proliferation and is upregulated in breast, colorectal, lung and other cancers. hnRNP A2 is also frequently overexpressed in various cancers (David and Manley, 2010). Increased hnRNPs can reduce apoptosis and increase proliferation. Another important splicing regulatory protein, PTB, has increased expression in ovarian cancers and glioma. The SR protein, SRSF1, has been investigated regarding its role in promoting cancer growth. SRSF1 has been classed as proto-oncogene and is associated with alternative splicing events that can alter cell motility and can transform immortalised cells *in vitro* (Karni et al., 2007).

Some of the changes to alternative splicing have functional activity in helping to promote cancer growth. Frequently, alternative splicing can create two proteins from the same gene with opposing activity. Cancer cells can use this feature to increase expression of a splice isoform that is beneficial for the tumour. A well-studied example of this is the proteins of apoptotic pathways. One of the important features of cancer cells is their ability to evade cell death through apoptotic pathways. Several genes that encode apoptotic regulatory proteins can produce isoforms with pro- or anti-apoptotic activity depending on alternative splicing of the pre-mRNA. For example, the protein Bcl-x(s) is pro-apoptotic but use of a alternative 5’ splice site in exon 2 of the pre-mRNA creates the protein Bcl-x(L), which inhibits cell death through apoptosis. Various cancers display high Bcl-x(L) to –x(s) ratio with increased Bcl-x(L) associated with higher risk of

metastasis and a reduced response to chemotherapy. The significance of Bcl-x splicing regulation was demonstrated by targeting oligonucleotide probes to Bcl-x RNA, increasing use of the splice site for the pro-apoptotic Bcl-x(s). Changing the ratio of Bcl-x(L) to –x(s) was able stimulate apoptosis in a prostate cancer cell line (Mercatante et al., 2002).

Alternative splicing is an important regulatory step in another of the cancer hallmarks, invasion and metastasis. As tumours grow they can acquire a more invasive phenotype by undergoing epithelial-mesenchymal transition (EMT). Transition of epithelial cells towards a mesenchymal phenotype involves changes in transcription, post-transcriptional modifications and alternative splicing. It is well established that the alternative splicing of fibroblast growth factor receptor II (FGFRII) transcripts is linked to EMT. FGFRII has two mutually exclusive alternative exons- exon IIIb and IIIc. Epithelial cells select exon IIIb to be included in FGFRII. During EMT there is a switch, with exon IIIc inclusion characteristic of mesenchymal phenotype. Many other changes in cellular function occur alongside the switch in IIIb/IIIc. The RNA binding proteins hnRNPA1 and PTB, which are known to be overexpressed in cancers, are involved in silencing exon IIIc (Yasumoto et al., 2004). Two key regulators of alternative splicing, ESRP1 and ESRP2, were identified by searching for genes involved in promoting IIIb splicing. ESRPs (epithelial splicing regulatory proteins) are expressed only in epithelial cells with expression lost during EMT. Overexpression of ESRPs in mesenchymal cells can reverse the splicing of FGFRII, silencing IIIc. ESRPs are thought to be tumour suppressors by acting as master regulators of the splicing decisions that define epithelial phenotype (Warzecha et al., 2009).

1.2.8 Using small molecules to modulate alternative splicing

With the implication of aberrant alternative splicing during various diseases established and the mechanisms of regulation becoming clearer, attempts are being made to find small molecules that can change alternative splicing patterns during disease to provide therapeutic benefit. Small molecule modifiers are also important during basic research as tools to further our understanding of the

biological processes that control splicing. Some chemotherapeutic drugs, such as cisplatin, also have an effect on alternative splicing. The splicing of several apoptotic genes are modified by chemotherapy, which may contribute to their anti-tumour activity (Shkreta et al., 2008).

Many small molecules shown to modify splicing directly target components of the spliceosome, inhibiting splicing in general but others have been found to indirectly modify alternative splicing patterns by interfering with upstream regulatory pathways. For example, amiloride is an inhibitor of sodium/hydrogen exchanger isoform 1 (NHE1). NHE1 is a membrane spanning ion transporter involved in regulating the volume and pH of the cell. Amiloride acts as a potassium sparing diuretic and as been used to treat hypokalaemia, oedema and as an anti-hypertensive. More recently, amiloride has also been found to effect alternative splicing in leukaemic cells. The *Bcl-x* and *HIPK3* gene are both apoptotic regulators, their transcripts can be alternatively spliced to create isoforms with either pro- or anti-apoptotic activity. Amiloride treatment of leukaemic cells increased the presence of pro-apoptotic transcripts. A genome wide exon array showed amiloride to also affect the alternative splicing of several other gene transcripts associated with apoptosis, including *SURVIVIN* and *APAF-1*. The effect of amiloride on alternative splicing may be mediated via changes in activity and/or expression of certain splice factors. SRSF1 expression and phosphorylation were reduced upon amiloride treatment, whereas hnRNP A1 expression increased. SR proteins are targets for phosphorylation by certain kinases and dephosphorylation by phosphatases. Amiloride treatment increased activation of the phosphatase, PP1. Reduction in SR protein phosphorylation by amiloride may be mediated by activated PP1 (Chang et al., 2011).

In another example, several small molecules have been identified with the ability to influence *SMN2* alternative splicing (Naryshkin et al., 2014). As described above, the SMN2 protein is non-functional due to a mutation in exon 7 of the gene, which promotes skipping of the exon leading to the protein product being

degraded. The neuromuscular disorder, SMA, occurs when the paralogue gene *SMN1* is deleted (Lewelt et al., 2012). There is currently no treatment that targets the cause of the disease. Disease severity depends on the levels of functional SMN protein produced with a 70% reduction in protein during severe type I SMA and 30% with mild type III SMA. Changing alternative splicing to increase the level of full length SMA may provide therapeutic benefit. A screen was performed using a large library of compounds and three small molecules identified as able to induce inclusion of exon 7 in *SMN2* transcripts, increasing levels of full-length protein in cells from SMA patients. Whole-transcriptome RNA sequencing demonstrated that these molecules are relatively specific, only changing splicing of a few pre-mRNAs. In a mouse model of severe SMA, the levels of full-length SMN were increased by treatment with the compounds. Motor functions of the mice were improved and the life span extended (Naryshkin et al., 2014). In a separate investigation, several analogues of cantharidin, a phosphatase inhibitor, were found to increase the inclusion of exon 7 in *SMN2* transcripts. Cantharidin and the analogues block the action of the phosphatase PP1 causing reduced phosphorylation of the splicing factor TRA2β and increased exon 7 inclusion (Zhang et al., 2011).

1.3 VASCULAR ENDOTHELIAL GROWTH FACTOR

1.3.1 VEGF

The regulation and co-ordination of angiogenesis is an intricate process with numerous growth factors, cytokines, signalling molecules and receptors involved. Vascular endothelial growth factor is an essential and the most intensively investigated regulator of angiogenesis. VEGF, also known as VEGF-A, is part of a family of growth factors, which include VEGF-B, -C, -D, and placental growth factor (PlGF). VEGF-C and VEGF-D have important roles in the regulation of lymphangiogensis whereas PlGF seems to only be important during pathology (Ferrara et al., 2003).

When Folkman proposed inhibiting angiogenesis as a method to treat cancer, he hypothesised that a diffusible factor produced by tumours promotes vessel growth (Folkman, 1971). This had previously been demonstrated *in vivo* as implanted melanoma cells were able to induce vessel growth even when a filter was applied between the tumour and the host (Ehrmann and Knoth, 1968). This diffusible molecule was partially isolated from tumour and able to induce neovascularisation in the dorsal air sac model (Folkman et al., 1971) and released by cultured tumour cells (Klagsbrun et al., 1976). In 1983, a protein that promoted microvascular leakage was shown to be released by several tumour cell lines. The protein was given the name vascular permeability factor (VPF) (Senger et al., 1983). Separately, Ferrara and colleagues isolated a mitogenic factor that could promote endothelial proliferation. Sequencing of the protein did not match any of the currently known angiogenic factors such as bFGF and was termed vascular endothelial growth factor. Sequencing of the cDNA encoding VEGF and VPF revealed them to be the same protein (Ferrara, 2002; Keck et al., 1989; Leung et al., 1989).

1.3.2 VEGF-A gene

Vascular endothelial growth factor A is encoded by the *VEGF-A* gene (Figure 1-5A). Alternative splicing of the gene's 8 exons creates several isoforms of varying lengths (Tischer et al., 1991). Exons 1-5 are constitutive, with alternative

splicing of exons 6-8 creating the different isoforms. The most commonly described are $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, the number indicates the amino acid length of the translated protein (Figure 1-5B, upper panel).

The longer isoforms, $VEGF_{189}$ and $VEGF_{206}$, contain amino acids encoded by exon 6 and bind to the cell surface and the extracellular matrix. $VEGF_{165}$ is partially secreted with a proportion sequestered by ECM binding. The ECM associated VEGF isoforms can be released to be active, diffusible fragments via plasmin cleavage at the protein's carboxy terminus. Isoforms that contain exon 7 sequence bind the glycosaminoglycan, heparin, with high affinity. $VEGF_{121}$ lacks all of exons 6 and 7 making it a freely diffusible protein isoform and does not have any association with heparin (Figure 1-5C). But association with heparin enhances the activity of VEGF as a mitogen (Ferrara et al., 2003). The loss of the C terminal or heparin binding ability of the protein significantly reduces the potency of VEGF as a mitogen (Keyt et al., 1996).

As described above, *VEGF* gene expression is regulated by hypoxia. Low oxygen reduces hydroxylation of the HIF transcription factors allowing them to evade degradation. Therefore, under hypoxic conditions HIFs promote transcription of various genes including VEGF. Additionally, many growth factors and cytokines have been shown to regulate VEGF expression alongside hypoxia. Paracrine or autocrine secretion of EGF, TGFα, TGFβ, IGF1, FGF, IL6 and more can induce VEGF expression in various cell types (Ferrara et al., 2003).

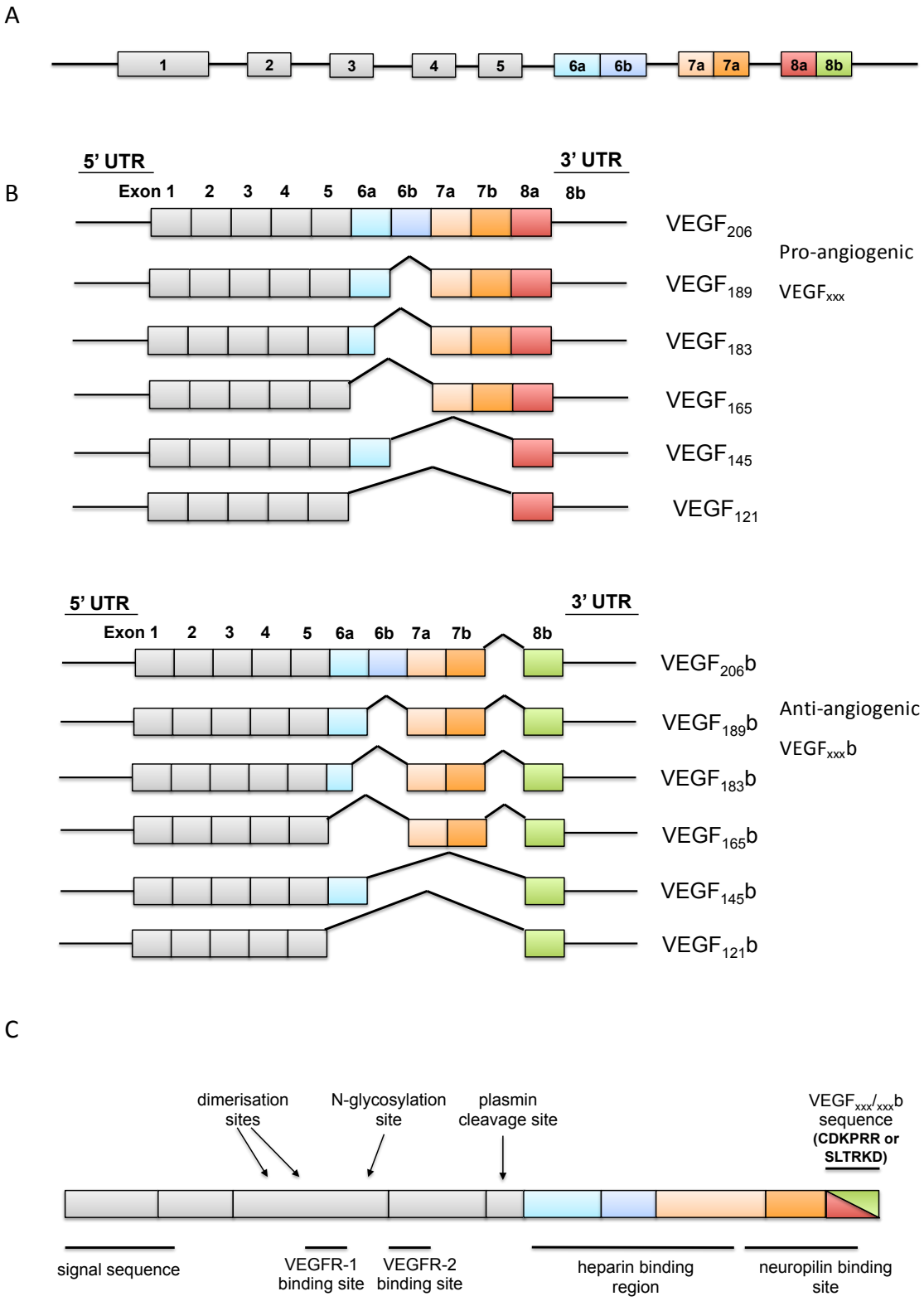


Figure 1-5 Structure of VEGF gene, mRNA and protein

(A) The human VEGF-A gene is located on chromosome 17. (B) Exons 1-5 are constitutively spliced. Alternative splicing of exons 6-8 creates many VEGF protein isoforms. Exon 8 has two alternative 3' splice sites, alternate use creates VEGF isoforms with pro- or anti-angiogenic activity. (C) Domains and features of VEGF protein.

1.3.3 VEGF receptors

The effects of VEGF are mediated via two cell surface receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. The extracellular portion of the receptors is made of seven Ig-like domains. A single transmembrane domain links to the intracellular domain, which has tyrosine kinase activity. VEGFRs commonly associate with neuropillins, a group of co-receptors.

The first VEGF receptor to be identified was VEGFR-1, which is also referred to as Flt-1. VEGF, PlGF and VEGF-B can all interact with VEGFR-1 but its exact role still requires investigation. A VEGFR-1 soluble isoform, created by alternative splicing, acts as an endogenous inhibitor of VEGF. Only weak tyrosine autophosphorylation is induced by ligand binding to VEGFR-1, leading to the hypothesis that VEGFR-1 doesn't primarily function as a signal transducer but as a decoy receptor to reduce the effect of VEGF on endothelial cells (Park et al., 1994). The importance of VEGFR-1 during embryogenesis has been shown by *Flt1*$^{-/-}$ knockout in mice, which causes embryonic lethality between 8.5 and 9.5 days. In VEGFR-1 deficient mice, endothelial tubes cannot form and mortality is caused by uncontrolled angioblast proliferation (Fong et al., 1999). This hypothesis is also supported by the finding that tyrosine kinase deficient VEGFR-1 is not lethal and does not cause vascular abnormalities in mice (Hiratsuka et al., 1998).

The majority of VEGF's effects are mediated via binding to and the subsequent signalling through VEGFR-2, which is also referred to as KDR or Flk-1. VEGFR-2 is crucial for functional angiogenesis during development. This is clearly demonstrated by the defects of mice lacking VEGFR2. Vasculogenesis and vessel organisation is impaired leading to death between days 8-9 in utero (Shalaby et al., 1995). VEGFR-2 proteins dimerize and ligand binding induces auto-phosphorylation of tyrosine residues. Various signalling molecules within endothelial cells are activated by VEGFR-2 stimulation, including Ras, PI3K and PLCγ.

Neuropillins (NRP1 and NRP2) are co-receptors involved in VEGF and VEGFR binding. NRP1 expression enhances the association between VEGFR-2 and $VEGF_{165}$. $VEGF_{121}$ does not interact with NRP1 which can rationalise why $VEGF_{121}$, even though freely diffusible, is not as potent a mitogen as $VEGF_{165}$ (Soker et al., 1998).

1.3.4 The role of VEGF in development and physiology

The *in vitro* and *in vivo* action of VEGF on endothelial cells and angiogenesis has been well studied. A strong angiogenic response is stimulated by VEGF in various *in vivo* models of angiogenesis (Leung et al., 1989). VEGF promotes the proliferation and survival of endothelial cells *in vitro* and *in vivo* (Carmeliet and Jain, 2011; Gerber et al., 1999)

The expression of VEGF is essential during embryogenesis. Embryonic lethality occurs at day 11-12 with a knock out of just a single *Vegf-a* allele. The other VEGF family members cannot compensate for the knockout with major developmental abnormalities observed in the embryos (Carmeliet et al., 1996; Ferrara et al., 1996). Whereas, homozygous knockout of *Vegf-b* or *pgf* does not cause any discernable developmental malformations.

VEGF was also shown to be crucial for early development and growth. Partial *Vegf* knockout was induced in newborn mice using Cre-loxP gene ablation. The knockout produced an increased death rate, reduced growth and diminished development of the organs, particularly the liver and kidneys. In further experimentation, administration of a soluble VEGFR-1 chimeric protein was used to inhibit VEGF in young mice. The inhibition of VEGF was lethal, causing endothelial deformities alongside renal and liver failure (Gerber et al., 1999). Likewise, anti-VEGF antibodies and deletion of *VEGF* in renal podocytes can cause glomerular dysfunction. Homozygous glomerular specific knockout of *Vegf* results in perinatal death whereas heterozygous knockout leads to profound renal disease within three weeks of birth (Eremina et al., 2003).

In the developed adult, angiogenesis only occurs in particular situations such as wound healing or in the female reproductive system. VEGF is highly expressed in the ovarian corpus luteum and needed of follicle development. Inhibitors of VEGF block the proliferation of vessels in the corpus luteum and disrupts follicular growth (Phillips et al., 1990).

1.3.5 The role of VEGF in disease

Expression of VEGF is high, but not uniform, in tumours. Ischaemic parts of tumours have the greatest VEGF expression, this observation lead to the discovery of hypoxia as a regulator of VEGF allowing vascular expansion into ischaemic areas (Plate et al., 1992). Anti-VEGF agents, such as VEGF antibodies and small molecule inhibitors of VEGF receptors, can inhibit the growth of cancer cells in many *in vivo* tumour models. Tumour and surrounding stromal cells secrete high levels of VEGF. VEGF activity is also enhanced by upregulation of matrix metalloproteinases, which release VEGF isoforms sequestered in the ECM via proteolyic cleavage.

Intraocular neovascularization and vessel leakage are characteristic of eye diseases such as diabetic retinopathy and age-related macular degeneration. Aqueous and vitreous humour of retinopathic eyes have increased levels for VEGF, with neovascularisation of the retina reduced by administration of soluble VEGFR-1 or VEGFR-2 protein in animal models (Aiello et al., 1995).

Many inflammatory conditions have links with VEGF. Epidermal keratinocytes produce high levels of VEGF during the process of wound healing and in psoriasis. Chronic inflammation in the skin can be caused by VEGF shown by targeted overexpression in the skin of transgenic mice. High density of cutaneous microvessels was observed with increased adhesion and rolling of leukocytes (Auerbach et al., 2003).

1.3.6 Anti-angiogenic VEGF isoforms

As described, there are various alternatively spliced VEGF isoforms of different length. Alternative splicing of exons 6 and 7 creates isoforms with high or weak affinity for the glycosaminoglycan heparin. In 2002, a previously unreported alternative splicing event in the terminal exon of VEGF pre-mRNA was described. An alternative distal 3' splice site (DSS) in exon 8 was found 66 base pairs downstream from the canonical proximal exon 8 3' splice site (PSS), splitting exon 8 into two sub-exons 8a and 8b. Selection of the exon 8 DSS creates a new open reading frame containing an equal number of nucleotides as with PSS use (Figure 1-5B, lower panel). Selection of this splice site results in a change of the six amino acids at the C terminus of the protein. This creates an alternative sister family of VEGF protein isoforms, which are given the names $VEGF_{xxx}b$, with $_{xxx}$ indicating the number of amino acids in the protein. The canonical $VEGF_{xxx}$ proteins contain sequence encoded by exon 8a resulting in the amino acids CDKPRR in the translated protein. Distal splice site selection causes $VEGF_{xxx}b$ isoforms to possess the unique sequence of SLTRKD encoded by exon 8b (Figure 1-7). The canonical $VEGF_{xxx}$ proteins and their sister isoforms have different biological properties caused by the change in amino acids at the C terminus (Figure 1-5C, Figure 1-7) (Bates et al., 2002; Woolard et al., 2009). Expression of $_{xxx}b$ versions of many VEGF isoforms has been detected in various tissues and cell lines, including $VEGF_{121}b$, $VEGF_{145}b$, $VEGF_{165}b$ and $VEGF_{189}b$ (Perrin et al., 2005). The sister isoform family have been shown to be anti-angiogenic, in contrast to the potent pro-angiogenic activity of $VEGF_{xxx}$.

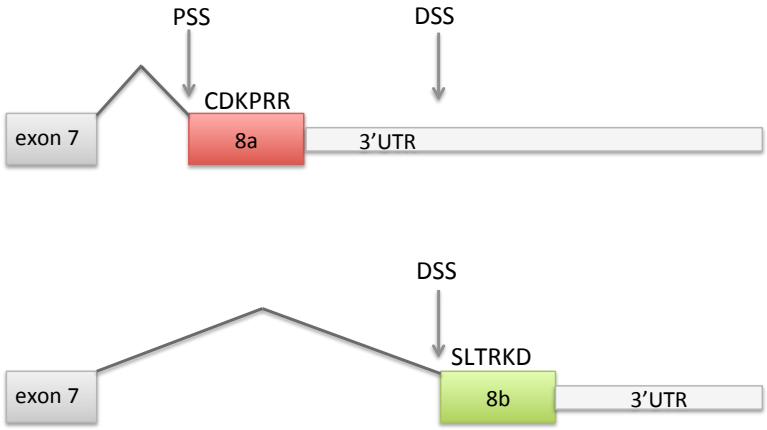


Figure 1-7 Alternative splicing of VEGF exon 8

VEGF exon 8 has two alternative 3' splice sites. Use of either splice site determines the final six amino acids of the translated VEGF protein.

1.3.7 The properties and function of $VEGF_{xxx}b$

The change in exon 8 3' splice site selection has consequences on the structure, interaction with receptors and functional properties of the resulting protein. The tertiary structure of $VEGF_{xxx}b$ proteins is altered, as they do not contain a key cysteine residue, Cys160, which is required to create a disulphide bond with Cys146 encoded by exon 7. Changes in the charge of the amino acids encoded by exons 8a/8b also affect the protein structure. The C terminus of $VEGF_{xxx}$ has two charged arginine residues, which are replaced by lysine and aspartic acid in $VEGF_{xxx}b$ isoforms (Bates et al., 2002; Woolard et al., 2009).

$VEGF_{xxx}b$ isoforms retain exons 3 and 4 present in the canonical isoforms. These are the sequences responsible for protein dimerization and binding to VEGF receptors. $VEGF_{xxx}b$ molecules can for homodimers and bind VEGFR-1 and VEGFR-2. Binding assays proved that $VEGF_{165}b$ binds to VEGFRs with the same affinity as $VEGF_{165}$ but the phosphorylation and activation of the receptor was reduced (Kawamura et al., 2008). Therefore, $VEGF_{165}b$ can act as a competitive inhibitor. The phosphorylation pattern induced by $VEGF_{xxx}b$ is different compared to the pro-angiogenic isoforms. $VEGF_{165}b$ binding does not cause phosphorylation of VEGFR-2 Tyr1054 which is found in the kinase regulatory

region of the receptor. VEGR-2 downstream signalling induces the phosphorylation of Erk, JNK and Akt. In comparison, the activation of these molecules is more transient and weaker when $VEGF_{165}b$ activates VEGFR-2.

The presence of $VEGF_{165}b$ and the other $_{xxx}b$ proteins has been detected in various tissues and cell lines. mRNA of $VEGF_{165}b$ transcripts was first identified via RT-PCR of renal cortex RNA. $VEGF_{xxx}b$ protein and/or RNA have since been found in primary cell lines such as retinal-pigmented epithelial cells and podocytes. In many human tissues $VEGF_{xxx}b$ isoforms comprises almost 50% or more of the total levels of VEGF, but this does vary significantly between different tissues. $VEGF_{165}b$ has been found in bladder, lung, urine, colon, kidney, vitreous fluid, prostate and many other human tissues (Bates et al., 2002; Bevan et al., 2008; Perrin et al., 2005).

$VEGF_{165}b$ protein has been shown to be anti-angiogenic *in vitro* and *in vivo* and does not increase the permeability of microvessels. $VEGF_{165}b$ does not stimulate the proliferation or migration of endothelial cells *in vitro*, and furthermore, inhibits the increased proliferation and migration induced by pro-angiogenic VEGF isoforms. $VEGF_{165}b$ can abolish vasodilatation promoted by $VEGF_{165}$ *in vitro* (Bates et al., 2002).

The anti-angiogenic role of $VEGF_{165}b$ in physiology has been investigated in several models. $VEGF_{165}b$ is highly expressed in human and mouse breast tissue with reduced expression when lactation occurs. A transgenic model was created whereby $VEGF_{165}b$ was overexpressed during mammary development. The mice have reduced vessel growth and less blood in the mammary tissue. Pups produced by the mice die as the mother cannot generate enough milk. This demonstrates that the down-regulation of $VEGF_{165}b$ is necessary for sufficient lactation and the $VEGF_{xxx}b$ proteins have an important role in normal physiology (Qiu et al., 2008).

As part of the rabbit corneal pocket assay, tumour cells are implanted in the cornea eliciting an angiogenic response. An increase in corneal angiogenesis was induced by breast cancer cells expressing $VEGF_{165}$ compared to control cells, whereas cells expressing $VEGF_{165}b$ did not increase angiogenesis. Furthermore, tumour cells overexpressing both isoforms, pro- and anti-angiogenic, reduced the angiogenic response compared to $VEGF_{165}$ alone. This demonstrates that $VEGF_{165}b$ can inhibit angiogenesis stimulated by pro-angiogenic VEGF isoforms. A similar effect was observed in other *in vivo* models of angiogenesis including a rat mesenteric angiogenesis assay (Woolard et al., 2004).

1.3.8 Anti-angiogenic VEGF isoforms and disease

Many diseases characterised by neovascularisation have been shown to be associated with altered levels of the anti-angiogenic VEGF isoforms. The level of $VEGF_{xxx}b$ was decreased in vitreous fluid taken from patients suffering from diabetic retinopathy and in the placenta of preeclamptic women. $VEGF_{xxx}b$ protein expression is decreased in many cancers compared to normal tissue including, renal cell carcinoma, colorectal cancer, melanoma and prostate cancer (Bates et al., 2002; Pritchard-Jones et al., 2007; Rennel et al., 2008; Varey et al., 2008b; Woolard et al., 2004).

The effect of anti-angiogenic isoforms on cancer cells and their influence on tumour growth has been investigated. The subcutaneous injection of LS174t colon carcinoma cells over-expressing $VEGF_{165}$ into nude mice caused the formation of large vascular tumours that were significantly bigger than tumours of control cells. Tumours formed by cells overexpressing $VEGF_{165}b$ were significantly smaller than control tumours. Also, tumour size was reduced when expressing both $VEGF_{165}$ and $VEGF_{165}b$ compared to only pro-angiogenic VEGF expression. A similar effect was seen in tumour models of renal cell carcinoma, prostate cancer and Ewing's sarcoma (Rennel et al., 2008; Varey et al., 2008b).

$VEGF_{165}b$ is anti-angiogenic and reduces cancer growth by inhibiting neovascularisation of tumours. The microvessel density observed in tumours is

decreases when overexpressing anti-angiogenic VEGF. $VEGF_{165}b$ expression decreases tumour growth *in vivo* but does not alter the proliferation of cancer cells *in vitro*, indicating the anti-tumour activity is caused by anti-angiogenic mechanisms. Media taken from cultured sarcoma cells secreting $VEGF_{165}b$ reduced the migration and proliferation of human microvascular endothelial cells (HMVECs) stimulated by $VEGF_{165}$ (Rennel et al., 2008).

1.3.9 Regulation of VEGF alternative splicing

VEGF exon 8 splice site selection is thought to be under the control of several SR proteins. Sequence analysis of the pre-mRNA revealed exon 8a to have a predicted binding site for SRSF1 upstream of the distal 3' splice site. Located downstream of the distal splice site was an SRSF6, also known as Srp55, predicted binding site. Overexpression of SRSF1 in cells promotes proximal splice site selection creating $VEGF_{165}$. Whereas SRSF6 overexpression increased $VEGF_{165}b$ transcripts (Nowak et al., 2008).

The action of these splice factors on VEGF pre-mRNA is subject to control by other regulators upstream. SRSF1 is a known target of the protein kinases SRPK1 and SRPK2. Inhibiting SRPK1 blocks phosphorylation of SRSF1 and reduces shuttling of SRSF1 to the nucleus upon stimulation. Blocking SRPK1 activity with small molecule inhibitors or knock-down of expression via RNAi switches the ratio of VEGF splicing, increasing anti-angiogenic isoforms. Interference with SRPK1 induces an anti-angiogenic effect *in vitro* and *in vivo* with reduced growth and microvessel density of tumours (Amin et al., 2011; Mavrou et al., 2014).

It is understood that like other gene expression regulatory mechanisms, alternative splicing can be modified by extracellular factors via signalling cascades. Various growth factors have been shown to affect the balance between VEGF pro- and anti-angiogenic isoforms through alternative splicing. IGF1 and TNFα favoured use of exon 8 proximal splice site, whereas TGFβ had the opposite effect and increased distal splice site use. Inhibiting SRPK1/2 or PKC reversed the effect of IGF1 on VEGF splicing. TGFβ induced distal splice site

selection was blocked by inhibiting p38 MAPK. This effect is thought to be mediated via CLK (CDC-like kinase) activation by p38 MAPK, which then phosphorylates SRSF6 promoting use of the distal splice site (Figure 1-8) (Nowak et al., 2008).

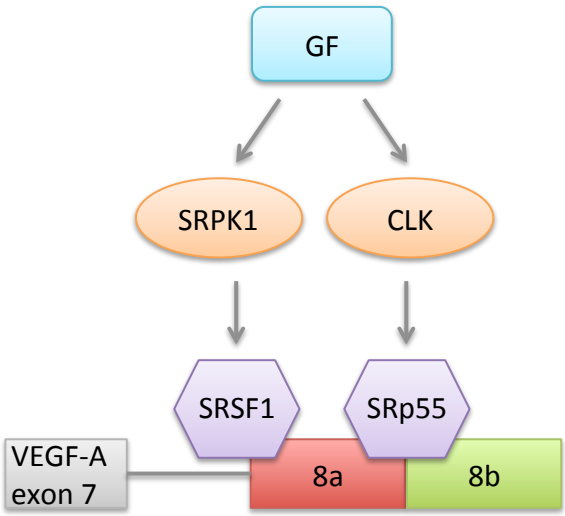


Figure 1-8 Regulation of VEGF exon 8 proximal and distal 3' splice site selection

Growth factors and other stimuli activate signalling pathways, which control the activity of regulatory kinases. Phosphorylation of splice factors can modulate their activity to promote use of different alternative splice sites.

1.4 HIGH-THROUGHPUT SCREENING AND DRUG DISCOVERY

High-throughput screening is a method that was first employed by pharmaceutical industry to aid drug discovery, with the aim to rapidly identify candidate chemicals that can be entered into the therapeutic research and development process. Screening has gained popularity due to its speed and relatively low cost. Miniaturisation, automation and robotics have all enabled the rapid expansion of screening methods (Pereira and Williams, 2007).

Drug discovery and validation is an exhaustive, lengthy procedure with many necessary stages. Screening is one of the initial stages following target identification, whereby thousands, even millions, of chemicals can be tested for inhibition of a specific protein or modulation of a cellular function as part of a quantitative bioassay. A large, diverse chemical library will be reduced to a few hit compounds, which can then be investigated further in lower throughput assays to confirm effect on the target. Once hit chemicals have been established, medicinal chemists can work to optimise the potency and pharmacokinetics of the chemical to provide better therapeutic potential. High-throughput screens have routinely been performed in a 96-well plate format. More recently, miniaturisation has enabled assays to be scaled up to a 384-well and sometimes 1536-well plate format, vastly increasing the number of chemicals that can be screened at once (Macarron et al., 2011).

Several different types of library can now be screened in bioassays including chemicals, cDNAs, siRNAs, proteins and peptides. Genome-wide cDNA and siRNA libraries are commonly used to elucidate which genes are important during a particular cellular process.

1.4.1 Bioassays for use in high-throughput screens

Assays used as part of a screen are normally either biochemical or use live cultured cells. Biochemical screens often look for inhibitors of a particular enzyme (e.g. kinases, proteases), agonists/antagonists of a receptor (e.g. GPCR) or a molecule that can interfere with a specific protein-protein association. This

is normally performed *in vitro* using purified proteins. However, chemicals identified during *in vitro* biochemical assays will not always demonstrate the same activity in a physiological setting; this could be caused by the chemical being unable to permeate the cell membrane, non-specific effects on other proteins or toxicity.

Assays that use cultured cells more closely reflect how potential lead compounds would act physiologically. A specific molecular target does not need to be defined for cell-based screens, with many assays looking for ways to alter a whole pathway or regulatory mechanism within the cell rather than inhibit a specific target. Also, such screens can be adapted for use with siRNAs or cDNA overexpression vectors to elucidate proteins important for the cellular function being investigated.

Various methods are employed to assay for the activity of small molecules in cells. But the chosen assay must be adaptable for a high throughput format. A functional bioassay commonly used to find inhibitors or activators of the G-protein coupled receptor superfamily (GPCRs) involves measuring levels of cAMP, which is produced as a second messenger upon GPCR activation. GPCR activation can be detected by measuring competition for binding to an anti-cAMP antibody between radiolabelled cAMP and cAMP endogenously produced by the cell. Only radiolabelled cAMP bound to the antibody is detected allowing quantification of cAMP production and therefore GPCR activation (Kariv et al., 1999).

Other bioassays aim to look for a change in cell phenotype or the disruption of a cellular process such as migration or proliferation. An example of such a screen is the high-throughput wound-healing assay developed by Yarrow et al. In this study, a wound was made in the monolayer of cells cultured in 384-well plates. 16,000 small molecules were tested for their effect on migration with an automated microscope used to image fixed cells and measure wound closure.

This screen identified a Rho-kinase inhibitor, which reduced formation of stress fibres required for migration (Yarrow et al., 2005).

Reporter gene assays have also been widely used in high-throughput screening. Commonly, expression plasmids are cloned with the whole or part of a gene of interest fused with the coding sequence of a reporter molecule such as lacZ, luciferase or a fluorescent protein. Detection of the reporter molecule provides information on gene expression or signalling to a specific promoter. The effect of a gene, siRNA or chemical from a screening library can be measured using various detection methods e.g. luciferase oxidation of luciferin, with emitted light measured using a luminometer.

1.4.2 Examples of high-throughput screening derived therapeutics

Drugs found by high-throughput screening have gone on to be used in human clinical trials with some eventually finding use as therapeutics. An early example a high throughput screening success is nevirapine, the anti-HIV agent. A compound library was tested for inhibitory action on HIV-1 reverse transcriptase with dipyridodiazepinones identified in as potent inhibitors. The pharmacokinetic properties were optimized and nevirapine shown to block HIV-1 reverse transcriptase activity and inhibit virus replication in culture. Nevirapine has been approved and used for the treatment of HIV-1 and AIDS for almost 20 years (Coster and Kumar, 2012; Merluzzi et al., 1990).

Another anti-retroviral used in HIV treatment, maraviroc, was developed as a result of a 500,000 compound library screen for inhibitors of CC-chemokine receptor 5. A ligand-binding assay was used to screen the library identifying an imidazopyridine as an agonist of the receptor. Work by medicinal chemists to alter the compound produced maraviroc, which antagonises the CC-chemokine receptor 5 causing inhibition of the membrane fusion process required for virus particles to enter a host cell (Dorr et al., 2005) . Similarly, chemical optimisation of a screen hit was used to create a drug for the treatment of thrombocytopenia (low platelet count). The thrombopoietin receptor is important for the activation

of JAK-STAT signalling required for megakaryocyte proliferation and platelet production. A screen for thrombopoietin receptor agonists was performed using a luciferase reporter under the control of a promoter activated by STAT. The final optimised compound, eltrombopag, was shown to increase platelet production and approved for use by the FDA to treat thrombocytopenia purpura (Erickson-Miller et al., 2005; Merli et al., 2015).

Cell based screens allow chemical modulators of a cellular process to be identified without a specific protein target in mind. For example, a compound screen identified a molecule that blocked the replication of hepatitis C virus. FRET was used to measure the activity of a viral protease giving an indication of viral replication. Analysis of virus strains that were resistant to the compound revealed that the molecule was an inhibitor of the protein NS5A protein, involved in hepatitis C RNA replication (Lemm et al., 2010; O'Boyle et al., 2005).

1.5 HYPOTHESIS AND AIMS

Angiogenesis and alternative splicing are the two processes focused on in this thesis. Vascular endothelial growth factor (VEGF) is a one of the most important signalling molecules associated with angiogenesis, and is an interesting protein with regards to its alternative splicing creating isoforms with opposing functions. Many studies have shown that $VEGF_{xxx}b$ proteins are anti-angiogenic compared with the strongly angiogenic $VEGF_{xxx}$ isoforms. Some of the pathways involved in regulating this splicing event have been elucidated.

Anti-angiogenic therapy has been intensively trialled as a treatment method for a variety of cancers with limited success. The vast majority of anti-angiogenesis drugs target VEGF or VEGF receptors. Inhibiting the action of all VEGF isoforms also blocks the anti-angiogenic effect of the $VEGF_{xxx}b$ family. As VEGF is highly expressed by cancer cells, switching alternative splicing of VEGF transcripts could increase expression of anti-angiogenic VEGF having an inhibitory effect on tumour angiogenesis and cancer progression. Some small molecule inhibitors of splicing pathways have already been shown to be potentially useful as anti-angiogenic/anti-cancer agents via altering *VEGF* terminal exon splicing (Gammons et al., 2013a; Gammons et al., 2014; Gammons et al., 2013b; Mavrou et al., 2014)

This project aimed to find molecules able to reduce angiogenesis by modifying *VEGF* alternative splicing. *VEGF* terminal exon alternative splicing was investigated using a novel approach, splicing-sensitive fluorescent reporters. Splicing reporters provide a way to investigate an alternative splicing event by following the expression of fluorescent proteins. A bichromatic VEGF splicing reporter (pRG8ab) was designed using the plasmid backbone of another splicing reporter and sequences from the *VEGF-A* gene.

The following aims were considered:

I. Validation of a splicing-sensitive fluorescent reporter designed to mimic alternative splicing of $VEGF_{xxx}$/$VEGF_{xxx}$b in culture.

II. Use of VEGF-based splicing-sensitive fluorescent reporters as a molecular screening tool: screening for small molecules that can alter *VEGF* terminal exon splicing promoting anti-angiogenic VEGF isoform production.

III. Test molecules which change *VEGF* splicing for anti-angiogenic activity *in vitro* and *in vivo*.

IV. Further investigate the activity of the small molecules to understand their mechanism of action in terms of VEGF splicing and anti-angiogenic activity.

Chapter 2

Materials and Methods

2.1 CELL CULTURE

2.1.1 Cell lines

Several different cell lines were used. HEK293 cells are a cell line derived from human embryonic kidney. PC3 cells are prostate cancer cell live derived from metastatic bone. Normal human dermal fibroblasts are derived from either adult skin dermis or neonatal foreskin dermis. HEK293, PC3 and NHDF cells sub-cultured from existing cultures within the lab. All were cultured in DMEM (D6429, Sigma) supplemented with 10% fetal bovine serum (10270, GIBCO) and 1% penicillin streptomycin (GIBCO). Human umbilical vein endothelial cells (HUVECs) were cultured in EBM-2 media (Lonza) supplemented with EGM-2 Bulletkit (Lonza). All cell lines were cultured at 37°C and in a humidified incubator with 5% CO_2.

2.1.2 Sub-culture

Cell lines were maintained in T75 cell culture flasks with growth medium refreshed every 2-3 days. Once cells reached ~80% confluency they were passaged at a ratio of 1:5- 1:10. To passage cells, the growth media was removed with an aspirator and the monolayer washed with 10ml 1xPBS. Cells were detached from the culture flask by adding 2ml of 0.5% trypsin-EDTA (Sigma-Aldrich) and incubation at 37°C for 4 minutes. Detachment was confirmed by examining cells under a light microscope. Trypsin was neutralised by addition of 8ml culture medium. The cell suspension was centrifuged for 3 minutes at 1,200rpm. The supernatant was aspirated from the cell pellet and cells were resuspended in 10ml culture medium. An appropriate number of cells were seeded into a T75 culture flask.

2.1.3 Thawing cells

Cryo-preserved cells were thawed in a 37°C water bath. Cells were transferred to a sterile tube and 10ml of serum rich media was slowly added to the cells. Cells were centrifuged for 3 minutes at 1200rpm. The supernatant was removed with an aspirator. The cell pellet was resuspended in 5ml normal growth medium with serum and transferred to a T25 culture flask.

2.1.4 Freezing cells

Cells were cultured in T75 culture flasks until ~80% confluent. Cells were washed, trypsinised and centrifuged as previously described. Each cell type was resuspended in 4ml of the appropriate culture medium containing 10% FBS and 10% DMSO (Fisher Scientific). 1.2ml of cell suspension was transferred to cryovials and stored at -80°C in an isopropanol chamber (Thermo Scientific) for 24 hours. For long-term storage, frozen cryovials were placed in liquid nitrogen tanks.

2.2 TRANSFECTION OF CELL LINES

2.2.1 Transient plasmid transfection

Cells were seeded into 6 well plates. When they reached ~80% confluency cells were transfected with plasmid. 2μg of plasmid was diluted in 100μl OptiMEM (GIBCO). 6μl of Fugene transfection reagent (Promega) was added to the solution to create a 1:3 ratio of plasmid:transfection reagent. The solution was mixed by shaking and incubated for 15 minutes at room temperature before being directly added to cells growing in 6 well plates. After 24 hours, successful transfection of fluorescent reporter plasmids was confirmed by fluorescence microscopy.

2.2.2 Creating stably transfected cell lines

After 48 hours, transiently transfected cells were washed with 2ml of 1xPBS and detached from 6-well plate using 0.5ml trypsin-EDTA and incubation at 37°C. 9.5ml of DMEM was added to tryspinised cells and they were transferred to a

T75 flask. G418 selection antibiotic was added to the culture media to allow selection for cells expressing the plasmid. Cells were cultured in selection antibiotic for 14 days to obtain stable plasmid transfection. 500µg/ml G418 was used to select in HEK293 cells. 750µg/ml was used to select PC3s.

2.3 FLOW CYTOMETRY

2.3.1 Analytical flow cytometry

The percentage of cells stably transfected with pRG8ab was quantified using flow cytometry. Trypsinised cells were counted using a haemocytometer and diluted with DMEM to give concentration of ~200,000 cells/ml. Cells were transferred to round-bottom FACS tubes. 4% PFA was added to the cell suspension to give a final concentration of 1% PFA. Fluorescence of the sample cells was measured and recorded using a BD FACS CantoII flow cytometer. Untransfected cells were first measured by the flow cytometer to gauge the background fluorescence produced by the cell type. The cytometer software was used to create a gate threshold around the background measurement. Fluorescent readings outside the gate were deemed to be true fluorescent signal produced by dsRED and/or within reporter-expressing cells.

2.3.2 Fluorescent activated cell sorting

FACS was used to sort fluorescent cells from the non-fluorescent population in cell lines stably transfected with fluorescent reporter plasmids. More than 20 million cells were used per sort. Cells were washed with 1xPBS, trypsinised and counted. Cells were centrifuged for three minutes at 1200 rpm. The media was removed from pelleted cells and they were resuspended in 1xPBS 25mM HEPES, 2.5mM EDTA, 0.5% BSA to give 5-8 million cells/ml. Cells in the buffer were transferred to round bottom FACS tubes (BD Falcon) that had been coated in fetal bovine serum. The cytometer (BD influx cell sorter) fluidics system moves cells through laser light sources in a single file manner. As each cell breaks off from the fluid stream, the droplet is given a charge. In this case, fluorescent reporter expressing cells were given the opposite charge to untransfected cells.

The opposing charges were used to separate the two populations. Charged deflection plates cause the charged droplets to be moved into separate collection tubes coated in fetal bovine serum. Sorted cells were centrifuged for 3 minutes at 1200 rpm and resuspended in DMEM supplemented with G418. Cells were cultured until confluent and fluorescent reporter expression was confirmed by analytical flow cytometry. Two sorts were performed on both PC3 and HEK293 reporter expressing cells to gain a high percentage of fluorescent cells within the population.

2.4 RNA WORK

2.4.1 RNA extraction

Cells were cultured in 6 well plates until confluency was reached. The cells were washed with 1xPBS. 1ml of Trizol Reagent (Life Technologies) was added to each well and the plate incubated at room temperature to allow lysis to occur. Lysates were transferred to sterile eppendorf tubes. 200μl of chloroform was added to each lysate and tubes shaken vigorously for 15 seconds before 15 minutes incubation at room temperature. The samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was separated from the organic phase and transferred to a new, sterile eppendorf. 500μl of isopropanol was added to each sample and mixed by shaking for 15 seconds followed centrifugation at 12,000rpm for 20 minutes at 4°C. The supernatant was separated from the pellet and discarded. 1ml of 75% ethanol used to wash the pellet and centrifuged for 5 minutes. The ethanol was removed and the pellet left to dry for a few minutes and subsequently resuspended in 20μl of DEPC water. The concentration of RNA in each sample was quantified using a Nanodrop spectrophotometer.

2.4.2 Treatment with DNase and cDNA synthesis

2μg of RNA was diluted with DEPC water to give a final volume of 8μl. RNA was treated with DNase to degrade any genomic DNA contaminants. 1 unit (U) of

RQ1 RNase-FreeDNase enzyme (M6101, Promega) was added to each reaction along with 1μl of 10x reaction buffer and incubated at 37°C for 1 hour. The addition of 1μl of RQ1 DNase Stop Solution and 10 minutes incubation at 65°C was used to stop the DNase reaction. The next step was to anneal DNA primers to the RNA template. 500ng of both oligo$(dT)_{15}$ (C1101, Promega) and random hexamer primers (C1181, Promega) were used. Primers were incubated with template at 95°C for 10 minutes and samples were then cooled on ice for 5 minutes. After primer annealing, the cDNA second filament was synthesised. 0.4mM dNTP mixture, 5μl M-MLV 5X reaction buffer and 200 units M-MLV reverse transcriptase (M1701, Promega) were added to each reaction and the total volume was made up to 25ul with DEPC water. cDNA production was completed via 90 minutes incubation at 37°C.

2.4.3 Reverse transcriptase PCR second strand synthesis

Polymerase chain reaction was used to amplify and demonstrate expression of pRG8ab plasmid (Figure 2-1) within transfected cell lines. Specific primers were designed to bind to the reporter plasmid artificial exon and dsRED sequences therefore amplifying transcripts splice at either the reporter proximal or distal splice site (reporter design detailed in Chapter 3). The expected size of PCR products was 193 and 127 base pairs for proximally and distally spliced transcripts respectively. 25μl of reaction mixture was made using 12.5μl of 2xPCR MasterMix (M7502, Promega), 0.8μM of forward and reverse primer mixture and DEPC water to bring the reaction to a volume of 23μl. 2μl of cDNA was added to each reaction and amplified using a thermal cycler. Primers used and cycling conditions are described in Table 1 Primer sequences and cycle conditions used for polymerase chain reactions**.** All PCR reactions included an initial step of 95°C for 5 minutes and final elongation step of 10 minutes at 72°C.

Alternatively spliced $VEGF_{165}$/$VEGF_{165}$b transcripts were also amplified from cDNA using PCR. For each reaction, 5μl of 5x Green GoTaq reaction buffer, 0,8μM forward and reverse primer mix, 0.2mM dNTP mix, 2μl cDNA and 1U

GoTaq DNA polymerase were made up to a final volume of 25µl with dH_2O. Table 1 lists the primer sequences and cycling conditions used.

PCR	Forward and reverse primers	PCR cycle conditions	Product sizes
pRG8ab	F: 5'-catatgccaagtacgccccctattgacg-3' R: 5'-ctacaggaacaggtggtggc-3'	40 cycles: 95°C for 30s 55°C for 30s 72°C for 60s	193 & 127bp
$VEGF_{165}$/ $VEGF_{165}b$	F: 5'-ggcagcttgagttaaacgaac-3' R: 5'-atggatccgtatcagtctttcctgg -3'	40 cycles: 95°C for 60s 55°C for 60s 72°C for 60s	153 & 57bp

Table 1 Primer sequences and cycle conditions used for polymerase chain reactions. All PCR reactions included an initial step of 95°C for 5 minutes and final elongation step of 10 minutes at 72°C

2.4.4 Quantitative RT-PCR

Quantitative RT-PCR was used to detect expression of mouse *Vegfr-2* as a marker of blood vessel presence in Matrigel plug in an *in vivo* angiogenesis assay. cDNA was made from extracted RNA as described. cDNA was diluted 1:3 with RNase/DNase free water. Primers specific for mouse *Vegfr-2* were used at a concentration of 425nM were used with 12.5µl of 2xSYBR Green Master Mix (Life Technologies) RNase/DNase and water to make 21µl reaction mixture. 4µl of cDNA was added to each reaction. PCR reactions were amplified using the Applied Biosystems StepOne Plus Real Time PCR System using the standard cycling mode: 95°C for 10 minutes followed by 40 cycles of 95°C 15 seconds and 60°C for 1 minute. Relative expression of mouse *Vegfr-2* was quantified by normalising to the expression of a reference gene, human GAPDH, using the

comparative Ct method. Reactions were set-up as described with GAPDH primers used at 1µM concentration.

ΔCt=Ct(target gene)-Ct(reference gene)

ΔΔCt=ΔCt(treatment group)-ΔCt(control group)

Fold change=$2^{(-\Delta\Delta Ct)}$

2.5 PROTEIN WORK

2.5.1 Protein extraction

Culture plates were placed on ice and washed with ice cold 1xPBS. A lysis buffer comprising of 1% NP-40, 150mM NaCl, 50mM Tris and 1x Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) was directly added to cells in culture plates. 50µl was used for each well of a six well plate. Cells in lysis buffer were scraped from culture plates and transferred to cold eppendorf tubes. Samples were kept on ice for 30 minutes with 30 seconds vortexing seconds every 5 minutes. Lysates were centrifuged at 4°C for 20 minutes at 12000rpm. Supernatant was collected and transferred to fresh, cold eppendorfs and stored at -20°C.

2.5.2 Western blotting

Protein samples were mixed with 5X protein loading buffer (National Diagnostics) and boiled at 100°C for 5 minutes. Samples were loaded into 4-15% Mini-PROTEAN TGX Stain-Free precast gels. A gel tank was filled with running buffer compromising of 25mM Tris-HCl, 192mM glycine and 0.1% SDS. Protein samples were separated by electrophoresis for around 1 hour at 150V. Exposure to UV in Gel Doc EZ (BioRad) was used to react trihalo compounds in the mini-PROTEAN TGX Stain-Free gels with Trp residues in separated protein samples producing fluorescence.

Separated proteins were transferred from the polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane using the electroblotting wet transfer method. PVDF was activated by incubation in methanol for 10 minutes before gel and membrane were placed in a transfer tank and covered with 25mM Tris-HCl, 192mM glycine, 20% methanol, 0.1% SDS transfer buffer. Tank was placed on ice and transfer performed at 90V for two hours. Total protein on the transferred membrane was visualised and imaged using Gel Doc EZ.

Membranes were blocked in either 5% BSA or 5% dried skimmed milk in TBS-Tween20 0.3% (TBS-T 0.3%). Blocking was performed for 1 hour at room temperature on a shaker. Membranes were probed with primary antibody diluted in TBS-T 0.3% overnight at 4°C. The next day, primary antibody was removed and membranes were washed with TBS-T 0.3% three times for five minutes. Membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (either anti-mouse IgG or anti-rabbit IgG) diluted 1:10000 in 2.5% dried skimmed milk TBS-T 0.3%. Membranes were incubated with secondary antibody for 1 hour at room temperature before washing in TBS-T 0.3% three times for five minutes. Clarity ECL Western Blotting Substrate kit (BioRad) was used to visualise detected proteins on the membrane. Membranes were imaged in Amersham Imager 600 (GE Healthcare).

Primary antibodies used and dilutions were: phosho-SR mab104 1:4, SR 1H4 1:1000 (sc-13509, Santa Cruz Biotechnology), $VEGF_{xxx}b$ 56/1 1:1000 (MAB3045-100, R&D Systems), VEGF A20 1:2000 (sc-152, Santa Cruz Biotechnology).

2.6 BACTERIAL TRANSFORMATION

Agar plates were made using LB broth with agar (L7533, Sigma-Aldrich). One sachet was dissolved in 500ml of dH_2O and was autoclaved to 121°C for 15 minutes to sterilize. After cooling to around 50°C, the agar was supplemented

with ampicillin at 100μg/ml. Agar was poured into Petri dishes and solidified at room temperature.

1μl of plasmid DNA was mixed with 30μl of DH5α competent cells (18258-012, Invitrogen) and placed on ice for 30 minutes. Bacteria were heat shocked at 42°C for 30 seconds and placed back on ice for 2 minutes. 300μl of S.O.C. Medium (15544-034, Invitrogen) was added to competent cells and incubated in a shaker for 45 minutes at 37°C. 50-200μl of bacteria in S.O.C. medium was pipetted onto an agar plate and spread across at agar surface. Plates were incubated overnight at 37°C.

20g of LB broth growth medium (L3022, Sigma-Aldrich) was suspended in 1L of dH_2O and autoclaved for sterilization. 5ml of LB broth containing 100μg/ml ampicillin was place in a sterile tube. A sterile pipette tip was used to touch a single transformed colony grown on the agar and placed in the LB broth. Lid were placed loosely on the tubes, which were incubated overnight in a 37°C shaker. Plasmid clones were extracted from transformed bacteria using the QIAprep Spin Miniprep Kit (27104, Qiagen).

2.7 CLONING OF CONTROL REPORTER PLASMIDS

2.7.1 PCR product generation

Polymerase chain reaction was used was used to amplify the DNA sequences from the pRG8ab plasmid. The primers used allowed amplification of fragments beginning at an NdeI restriction site at the 3' and ending at the exon 7-intron 7 junction. Two different reverse primers were used to create the fragments- one hand an overhanging region complementary to the proximal splice site and the other the distal splice site. These were termed Fragment A-PSS and Fragment A-DSS. Two more fragments were produced beginning at either the proximal or distal splice site and ending at a SacII restriction site. In this instance the forward primers had overhang complementary to exon 7. These fragments were termed Fragment B-PSS and Fragment B-DSS (Figure 2-1). Amplification reactions were

performed using Pfu polymerase (Thermo Scientific). Primers and cycling temperatures used are described in Table 2. One 50µl reaction was made as follows; 5µl Pfu 10x buffer, 1.25mM dNTP mix, 0.5µM forward and reverse primers, 1µg of pRG8ab plasmid and 2U Pfu polymerase. Resulting products were run on 2% agarose gels and extracted from agarose using QIAquick gel extraction kit (28704, Qiagen). Extracted fragments were cleaned up using QIAquick PCR purification kit (28104, Qiagen) and 5µl of each run on a 2% agarose gel to confirm efficient gel extraction.

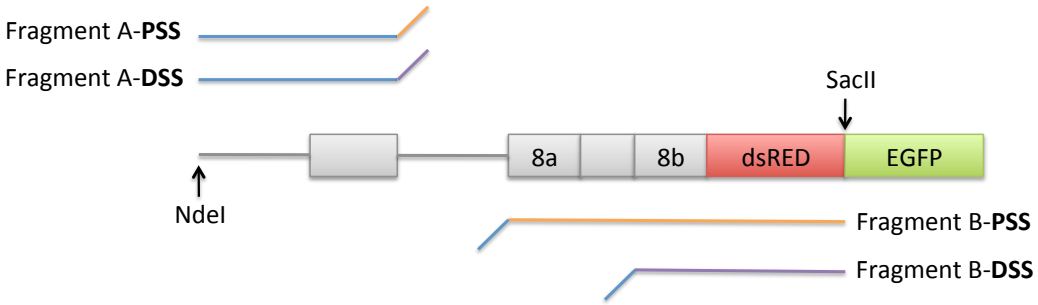


Figure 2-1 Fragments for control reporter constructs were amplified from pRG8ab plasmid.
Primers used to amplify the fragments possessed complementary overhanging regions to allow joining of the fragments by PCR overlap extension

PCR product	Forward and reverse primers	PCR cycle conditions	Product size
Fragment A-PSS	F: 5'-cgcatatgccaagtacgccccctattgacg-3' R: 5'-ctcggcttgtcacatctgcaagtacgtctagatcc-3'	40 cycles: 95°C for 30s 60°C for 30s 72°C for 2 minutes	525bp
Fragment A-DSS	F: 5'-cgcatatgccaagtacgccccctattgacg-3' R: 5'-ttcctggtgagagatctgcaagtacgtctagatcc-3'	40 cycles: 95°C for 30s 60°C for 30s 72°C for 2 minutes	525bp
Fragment B-PSS	F: 5'-tagacgtacttgcagatgtgacaagccgaggcggtg-3' R: 5'-cgccgcggtctacaggaacaggaacaggtggtggc-3'	40 cycles: 95°C for 30s 70°C for 30s 72°C for 2 minutes	788bp
Fragment B-DSS	F: 5'-tagacgtacttgcagatctctcaccaggaaagact-3' R: 5'- cgccgcggtctacaggaacaggaacaggtggtggc-3'	40 cycles: 95°C for 30s 70°C for 30s 72°C for 2 minutes	723bp

Table 2 Primer and PCR conditions used to create control reporter fragments. Each reaction was initiated with 5 minutes and 95°C and final 10 minute elongation step at 72°C.

2.7.2 Overlap extension PCR

Overlap extension PCR was used to create longer fragments from the two shorter templates that had been amplified as described above. This was performed in two stages- during the first 15 PCR cycles, no primers were used as the templates were originally generated using primers that have overhanging regions complementary to the other fragment that it is to join (Figure 2-1). This allows the two fragments to anneal at these overlapping regions and act as primers. After 15 cycles, in the second stage, 1μM mix of forward and reverse primers

that anneal to each end of the template (restriction sites) were added to the reaction and PCR continued for another 20 cycles. One reaction was made up as follows; 100μg of each template, 2.5U Pfu polymerase, 2.5μl 10x Pfu buffer plus DEPC water to a final volume of 25μl. 15 cycles were performed with denaturing for 30 seconds at 95°C, annealing for 30 seconds at 60°C and elongation for 2 minutes at 72°C.

2.7.3 Ligation into plasmid vector

PSS/DSS fragments and plasmid were cut using the restriction enzymes SacII and NdeI (New England Biolabs). Each 60μl reaction contained 6μl 10x cut smart buffer, 60 units SacII, 60 units NdeII and either 2μg plasmid or 50μg of fragment (PSS or DSS). Reactions were incubated for 3 hours at 37°C. The digested plasmid was run on a 1% agarose gel to separate the vector from the insert and the vector cut and isolated from the gel using QIAquick gel extraction kit.

The PSS and DSS inserts were ligated into to pRG vector using T4 DNA ligase. 10μl reactions were made with 400 units T4 ligase, 1μl 10x T4 ligase buffer, 3μl insert, 1μl vector and 4μl DEPC H_2O and incubated at room temperature for 1 hour. A control reaction performed with no DNA ligase. 1μl of each reaction was used to transform competent cells as described above.

In cases that direct ligation pRG plasmid vector and insert was unsuccessful, a Zero Blunt TOPO PCR cloning kit (450245, Invitrogen) was used. PCR products were inserted into the TOPO cloning vector as described in manufacturers instructions.

2.7.4 Site-directed mutagenesis

Errors in plasmid sequences were fixed using site-directed mutagenesis. Plasmid DNA was amplified using two complementary primers containing the desired mutation in the middle and Pfu Turbo DNA polymerase. For each reaction, 5μl 10x Pfu reaction buffer, 50ng plasmid template, 125ng of each primer, 0.5mM

dNTP mix, 2.5U Pfu Turbo were made up to a final volume of 50μl with dH_2O. Plasmid was denatured for 30 seconds at 95°C, primers annealed for 1 minute at 55°C and an extension step of 10 minutes at 68°C. This reaction was cycled 18 times. After cycling, 10U of Dpn1 restriction enzyme (R0176S, New England Biolabs) was added to each reaction and incubated at 37°C for 1 hour to digest the original methylated template DNA. 1μl of Dpn1 treated reactions were used to transform competent cells and mutated plasmid was isolated as described above.

2.8 CHEMICAL SCREEN

2.8.1 LOPAC primary screen

A library containing 1280 FDA-approved chemicals, LOPAC, Library of Pharmaceutically Active Compounds (LO4200, Sigma-Aldrich) was used. Chemicals in the library were dissolved DMSO at a stock concentration of 10mM. PC3 pRG8ab cells were trypsinised and diluted to $8x10^4$ cell/ml using DMEM. 100μl of the cell solution was seeded into each well of a black 96-well plate.

All compounds of the LOPAC were dissolved in DMSO. Each was diluted from the stock concentration of 10mM to 300μM using DMSO. The cells in each 96-well plate were treated with DMSO only and 16 different compounds from the LOPAC in triplicate at 10μM. Wells of the outside edges of 96-well plates were not used for treatments or measured for fluorescence. Plates were incubated at 48 hours at 37°C. Changes in reporter alternative splicing caused by treatment with LOPAC compounds were measured using a VICTOR X multi-label plate reader (Perkin Elmer). Different sets of narrow band excitation and emission filters were used in the plate reader to detect dsRED and EGFP expression. dsRED was measured using 550nm excitation and 632nm emission filter. Excitation 485nm and emission 535nm filters were used for EGFP detection. Each plate was measured using VICTOR plate reader three times. DMSO treated wells were used as control

measurements and statistically compared to treated wells from within the same 96-well plate using a one-way ANOVA.

2.8.2 Elimination of false positives in the control screen

Potential hit compounds identified in the primary chemical screen were used to treat two PC3 cell lines stably transfected with control reporters for pRG8ab proximal (dsRED) and distal (EGFP) splice site selection (PSS control and DSS control). 8,000 PSS control or DSS control cells were seeded into black 96-well plates. Cells were treated with 10μM of each compound for 48 hours before fluorescence was measured using VICTOR. The same parameters were used to measure dsRED and EGFP as in the primary screen. Differences between each treatment and control wells within the same plate were analysed by one-way ANOVA and Dunnett's post-test. Treatments that increased EGFP or decreased dsRED were considered false positives and eliminated from the screen.

2.9 *IN VITRO* CELL ASSAYS

2.9.1 Endothelial cell tube formation assay

HUVECs at passage 6 or lower were grown in 6 well plates until ~70% confluent. HUVECs were cultured in EBM-2 cell culture medium including the serum and growth factors of the EGM-2 bullet kit with the exception of VEGF. Cells were treated with ESSO compounds or controls for 48 hours before assay.

50μl of Matrigel basement membrane matrix (734-1100, VWR) was seeded into the appropriate number of wells of a chilled 96-well plate. The plate was placed at 37°C for >30 minutes to solidify the Matrigel. Pre-treated HUVECs in 6-well plates were detached from culture plates using trypsin-EDTA and counted using a haemocytometer. The cells were diluted using EBM-2 to a concentration of 200,000 cells/ml. 50ul of treated cells were seeded into wells of the 96-well plate onto the solidified Matrigel. The HUVECs were incubated at 37°C for 8 hours. Treatment with ESSO compounds and controls was continued during this period. Cells were imaged using phase contrast at a magnification of 10x. Five images

were captured per well. Tubule length and number of branch points were quantified for each image using ImageJ software.

In a similar experiment, PC3 cells were cultured in EBM-2 media and treated with chemicals at 10µM concentration. Following 48 hour treatment, the conditioned media was removed and stored at -20°C. Matrigel was used to coat the bottom of a 96-well plate as described above. 10,000s HUVEC were diluted in each of the conditioned media taken from compound-treated PC3 cells and seeded into Matrigel-coated wells. Cells were incubated and 37°C for 8 hours and imaged.

2.9.2 Angiogenesis co-culture assay

PC3 cells were cultured in T75 culture flasks. When 70-80% confluent, the media was removed and replaced with EBM-2 endothelial cell media containing ESSO compounds, DMSO control or 1nM $VEGF_{165}$ recombinant protein. After 48 hours, the conditioned media was removed from the PC3s and stored at -20°C.

An 11-day protocol was performed with endothelial cells co-cultured with confluent fibroblasts. Day 1-normal human dermal fibroblasts (NHDF) were cultured in DMEM supplemented with 10% FBS. Confluent NHDF cells were harvested with trypsin and counted using a haemocytometer. Cells were diluted in EBM-2 endothelial media to produce a concentration of $3x10^4$ cells/ml. 1ml of the cell suspension was seeded onto sterile coverslips in 12 well plates and incubated at 37°C. Day 4- media was removed from NHDFs and replaced with fresh EBM-2. Day 5- human umbilical vein endothelial cells (HUVECs) were diluted to $3x10^4$ cells/ml in EBM-2. The media on the fibroblasts in 12 well plates was removed and replaced with 1ml of the cell solution. Day 7- EBM-2 was removed and refreshed. Day 9- EBM-2 was removed from cells and replaced with the conditioned media taken from PC3s treated with ESSOs. Day 11- cells were fixed and stained for endothelial markers via immunofluorescence.

2.9.3 Trypan Blue cell viability assay

Cells were washed and trypsinised and described previously and diluted to an approximate concentration of $2x10^5$ cells per ml. 0.5ml of cell suspension was transferred to a screw cap tube and mixed thoroughly with 100µl of 0.4% Trypan Blue stain. Cells and the stain were incubated for 5 minutes at room temperature. A haemocytometer was filled and used to count cells under a microscope. Stained cells were non-viable and unstained that had excluded the stain were viable.

2.10 CD31 IMMUNOFLUORESCENCE

The media was removed from the cells and the coverslips were washed with 1ml 1xPBS and fixed for 15 minutes in 1ml 4% paraformaldehyde. After fixation, coverslips were washed twice with 1xPBS. Non-specific binding of antibodies was blocked by incubating with 3% bovine serum albumin (BSA) 1xPBS overnight at 4°C. The co-culture was stained for endothelial cell marker CD31 (PECAM). Monoclonal CD31 primary antibody (3528, Cell Signalling Technology) was diluted 1:500 in 1% BSA and the coverslips stained for 1 hour. Two washes in 1xPBS were performed before staining with Alexa Fluor 488 rabbit anti-mouse antibody. The fluorescent secondary antibody was diluted 1:1500 in 1% BSA 1xPBS and coverslips were stained for 1 hour. The co-cultures were washed two final times with 1xPBS and mounted onto microscope slides with Vectashield mounting medium with DAPI (H-1200, Vector Labs) to visualise the nuclei.

2.11 *IN VIVO* MODELS

2.11.1 Matrigel plug angiogenesis assay

PC3 cells were cultured in media containing 10µM a potential anti-angiogenic compound or DMSO for 48 hours. Cells were detached from culture plates and diluted in DMEM to a concentration of $2x10^7$/ml and placed on ice. Matrigel basement membrane protein (734-1100, VWR) was thawed on ice. 100µl of cell suspension was mixed with 400µl of Matrigel and 10µM of the test compound. The mix of cells and matrix was subcutaneously injected into each upper flank of

Crl:CD1-*Foxn1*^{nu} nude mice (Charles River). Four days post-injection, mice were culled by cervical dislocation (Schedule 1) and the Matrigel plugs were extracted. Images of each plug were taken and their colour quantified using Photoshop as an indication of blood vessel infiltration into the matrix. Plugs were flash frozen in liquid nitrogen. 1ml of Trizol was added to each plug and samples were homogenised for RNA extraction as described.

2.11.2 Subcutaneous xenograft models

The same procedure was followed as described above during the Matrigel angiogenesis assays. Two weeks following subcutaneous injection, mice were culled by cervical dislocation (Schedule 1) and tumours were extracted. Tumours were weighed and frozen.

2.12 STATISTICAL ANALYSIS

Comparisons of two datasets were performed using Students' t-test or a Mann Whitney U-test. A comparison of three or more groups was performed using one-way analysis of variance (ANOVA) with Dunnett's post-test. $P<0.05$ was considered to indicate a statistically significant difference.

Chapter 3

Construction and validation of the VEGF splicing-sensitive fluorescent reporter

3.1 INTRODUCTION

Several studies have utilised fluorescent splicing reporters to screen for splicing modulators. Initially monochromatic splicing reporters were commonly used to investigate interesting splicing events (Kemp et al., 2005; Moore et al., 2010; Oltean et al., 2006). In such reporters, expression of a fluorescent protein would be caused by inclusion or exclusion of an alternatively spliced region within an mRNA transcript. For example, Oltean *et al* developed a fluorescent minigene to investigate epithelial to mesenchymal transitions (EMT). The reporter was based on the alternative splicing of fibroblast growth factor receptor 2 (FGFR2) mRNA. FGFR2 transcripts contain two mutually exclusive exons- exon IIIb is included in epithelial cells whereas in mesenchymal cells, IIIb is silenced and exon IIIc is included. The pRIIIcl^2 reporter was used to follow the inclusion of the mesenchymal IIIc exon- the design of the reporter was such that inclusion of IIIc disrupts the open reading frame of RFP, therefore epithelial cells express RFP and the fluorescence is lost in mesenchymal cells.

Moore *et al* made use of two different monochromatic reporters to explore the alternative splicing of Bcl-2 family of apoptotic regulatory proteins. The mRNA transcripts of Bcl-2 like proteins can be alternatively spliced to give long or short isoforms, which are anti- and pro-apoptotic respectively. Two reporters were designed based on the Bcl-x and Mcl-1 genes with open reading frame and intronic regions fused to Venus or mCherry sequences. A premature stop codon was inserted into the alternatively spliced region, such that only short transcripts, which mimic pro-apoptotic variants, allow translation of fluorescent

proteins. These reporters were used to uncover novel modulators of pro- and anti-apoptotic splice variants via a genome-wide siRNA screen.

In addition to fluorescent proteins, luciferase reporters have been utilised in small molecule screens like for instance in SMN splicing. Due to alternative splicing only a small quantity of functional SMN protein is produced from the *SMN2* gene. Loss of SMN expression via mutation of the paralogous gene *SMN1* causes spinal muscular atrophy. A splicing reporter screen was used to elucidate small molecules that could alter alternative splicing of SMN2 transcripts to increase expression of functional SMN protein. The reporter was designed in such a way that the luciferase coding sequence was in frame only when an alternative exon was included in the reporter transcripts. Monochromatic reporters have been useful investigating alternative splicing especially in the context of screening and following splicing decisions *in vivo* but they do have limitations. A drawback to using monochromatic reporters is that they only allow quantification of one alternatively spliced transcript- changes cannot be compared to the other possible alternative splicing patterns (Naryshkin *et al*., 2014).

A bichromatic splicing-sensitive fluorescent reporter (SSFR), RG6, was constructed by (Orengo et al., 2006). This system allows two patterns of alternative splicing to be followed from the same reporter (Figure 3-1). The design, crucially, relies on the fact that the fluorescent protein dsRED does not have any translational stop codons in its +1 reading frame. The alternative splicing event exploited in the original reporter was skipping of exon 5 of chicken cardiac troponin T (cTNT). dsRED and EGFP coding sequences were positioned downstream of cTNT exon 5 and its surrounding introns. Skipping of the alternative exon completes the open reading frame for dsRED. The alternative exon inclusion causes a frame shift; it has 28 nucleotides, not a multiple of three. dsRED+1 is translated and a fusion protein with EGFP is formed.

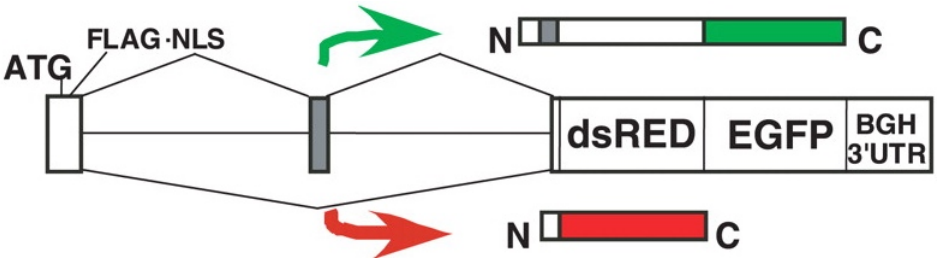


Figure 3-1 RG6 bichromatic fluorescent reporter designed by Orengo et al
The reporter minigene begins with an artificial exon and can be adapted to include a cassette or alternative 3' splice sites of interest allowing productions of two different fluorescent proteins upon selection of different alternative splice sites (Figure from Orengo et al.)

A similar bichromatic reporter construct was used to investigate inclusion or exclusion of exon 10 in MAPT (microtubule associated protein tau) transcripts. Frontotemporal dementia is linked with changes in MAPT splicing. Exon inclusion in the construct creates the correct reading frame for RFP translation whereas GFP is translated when exon 10 is spliced out of the construct (Stoilov *et al.*, 2008).

Dual colour reporters such as these can be utilized to evaluate the ratio of transcripts produced as a result of an alternative splicing event. An important feature of bichromatic reporters is that they allow measurement of an increase in one fluorescent protein and decrease in the other. This conveys greater sensitivity and dynamic range of the assay compared to a monochromatic reporter construct.

A bichromatic SSFR was designed to mimic the alternative splicing event of *VEGF* exon 8, which results in expression of either pro- or anti-angiogenic VEGF protein isoforms. It was aimed to validate the reporter for use in culture and to determine it's usefulness as a molecular screening tool for the identification of *VEGF* alternative splicing regulators.

3.2 RESULTS

3.2.1 Cloning and design of VEGF reporter

The VEGF SSFR design was based on the bichromatic splicing reporter, pRG6. The RG6 plasmid contains conveniently placed restriction sites that allow insertion of different alternatively spliced sequences. The function of the reporter relies on the inclusion or skipping of a variable region. In the case of the VEGF reporter, the variable region came from selection of either the proximal or distal 3' splice site for exon 8. The relevant sequences of the *VEGF* gene were amplified from genomic DNA. PCR amplification produced a 2,699bp DNA fragment containing the last 11 bases of *VEGF* exon 7, all of intron 7, exon 8a and 8b. The *VEGF* fragment was cloned into the RG6 backbone between XbaI and AgeI sites, the resulting reporter plasmid was assigned the name pRG8ab (Figure 3-2).

The expression of the construct is under control of a CMV promoter and the coding region begins with an artificial exon sequence from RG6. The artificial exon contains the XbaI restriction site- this is where the fragment amplified from *VEGF* begins. The endogenous *VEGF* region of the reporter ends at the AgeI restriction site. dsRED and EGFP coding sequences immediately follow. Crucially, the stop codons of exons 8a and 8b have been mutated to allow translation of the fluorescent proteins downstream. As previously described, the reporter function relies on dsRED and EGFP being in mutually exclusive reading frames.

Pro-angiogenic VEGF protein isoforms are produced when exon 8's proximal 3'splice site is used. Distal splice site selection leads to expression of anti-angiogenic VEGF. pRG8ab was designed to cause expression of dsRED or EGFP depending on splice site selection. During reporter pre-mRNA splicing, if the PSS is chosen, both the 8a and 8b regions of exon 8 are included in the mature transcript. Under these circumstances, dsRED is in-frame during translation. The out of frame GFP sequence is not translated due to a stop codon at the end of the dsRED open reading frame. A different reading frame is used when the DSS is chosen. In this case, the 8a region is not included in the mRNA transcript which

causes a frame shift. Exon 8b is translated but the dsRED sequence is out of frame leading to dsRED+1 being translated. A fusion protein is formed with EGFP, which is in-frame. Essentially, the pro-angiogenic splicing pattern causes dsRED expression, while EGFP is expressed by the anti-angiogenic splicing pattern.

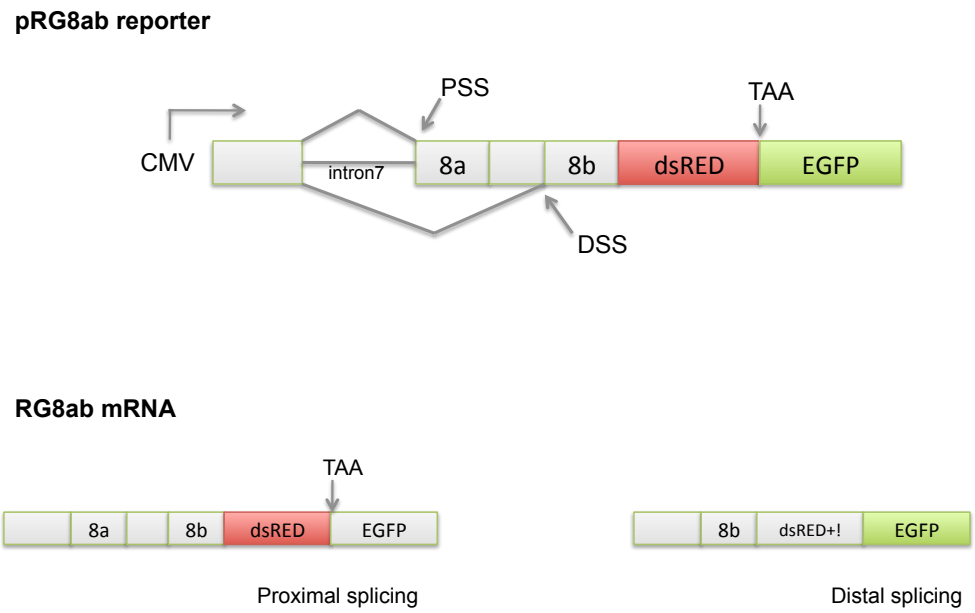


Figure 3-2 pRG8ab splicing-sensitive fluorescent reporter design
A VEGF splicing reporter was designed based on the RG6 construct. VEGF exon 7 3'end,, intron 7 and exon 8 were ligated into RG6 plasmid backbone. Depending on reporter 3' splice site selection, either dsRED or GFP is translated.

3.2.2 Reporter expression in culture

It was first determined as to whether the pRG8ab construct was well expressed with functional fluorescent protein production when tranfected into cultured cell lines. Human embryonic kidney cells (HEK293) were transiently transfected with reporter plasmid via lipofection. Reporter expression was confirmed by fluorescence microscopy 48 hours after transfection. Cells were fixed in 4% paraformaldeyde and the nuclei stained with Hoechst. The cell population had a transfection efficiency of ~50%. Expression of both dsRED and EGFP was

observed; this indicated that it is possible for the reporter RNA to be spliced from both the proximal (dsRED) and distal (EGFP) splice sites. Many cells expressed both dsRED and EGFP whereas other expressed only one of the fluorescent proteins (Figure 3-3). EGFP was localised mainly in the nucleus due to a nuclear localisation sequence within the in-frame EGFP sequence.

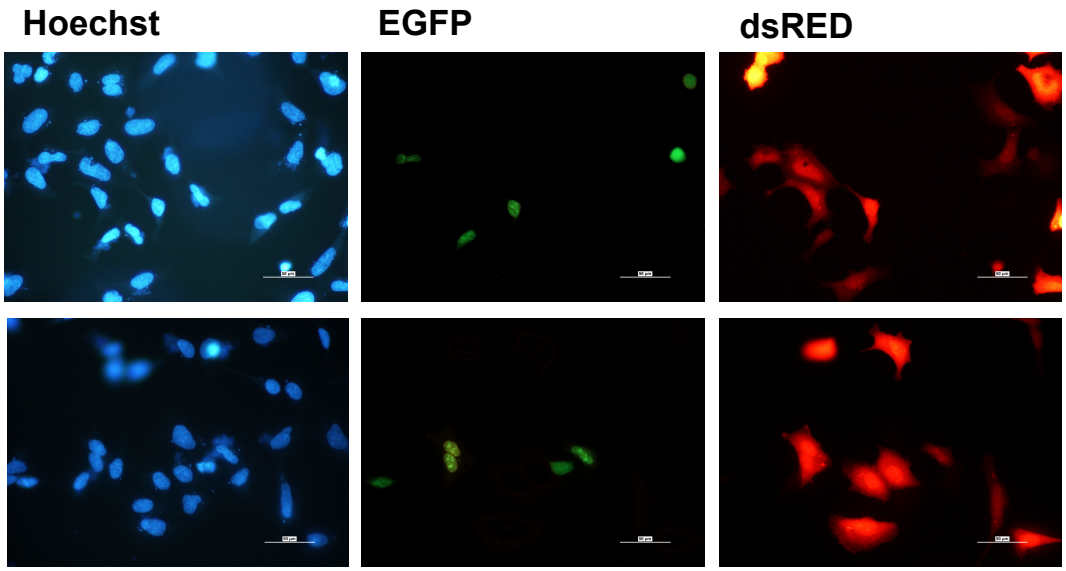


Figure 3-3 HEK293 cells transiently transfected with pRG8ab reporter Expression of both dsRED and EGFP was observed 48 hours after reporter transfection into HEK293 cells. Cells were fixed in 4% PFA and nuclei stained with Hoechst.

The reporter plasmid contains a *neo* resistance gene. Therefore, transfected cells were selected for using the aminoglycoside antibiotic, G418. Transiently transfected cells were cultured in growth media containing 500ug/ml G418 for 14 days. G418 is toxic to eukaryotic cells, only cells expressing the resistance gene survived the selection, to produce a HEK293 cell line that stably expressed pRG8ab. Following antibiotic selection, the cells were again imaged via fluorescence microscopy. The majority of transfected cells now expressed only dsRED, with little EGFP detectable (Figure 3-4). This corresponds with the

alternative splicing pattern of endogenous *VEGF* RNA in this cell type, as HEK293 cells predominantly express pro-angiogenic VEGF isoforms, as demonstrated by RT-PCR (Figure 3-5). On the other hand, cells transiently transfected with the reporter displayed both patterns of reporter alternative splicing. This is likely due to each transfected cell having taken up many copies of the reporter plasmid. This high copy number may overwhelm the splicing machinery configuration at one particular splice site and cause changes to the ordinary alternative splicing pattern.

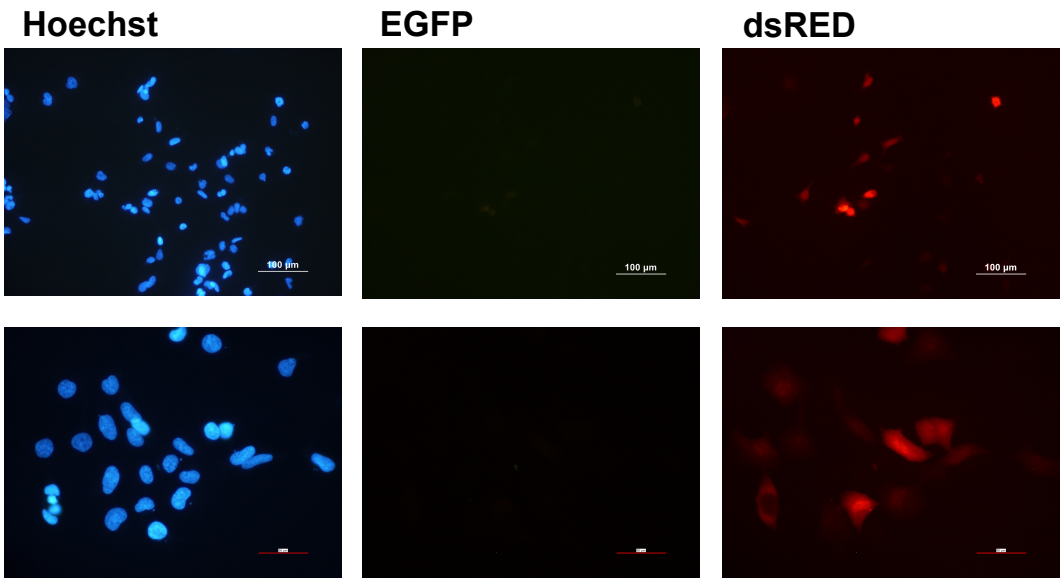


Figure 3-4 Stable transfection of pRG8ab in HEK293 cells
The reporter plasmid contains a neo gene which conveys resistance to G418 antibiotic. Transiently transfected cells underwent antibiotic selection (500ug/ml) to produce a stable cell line expressing pRG8ab. No EGFP expression was detected in the stable cells.

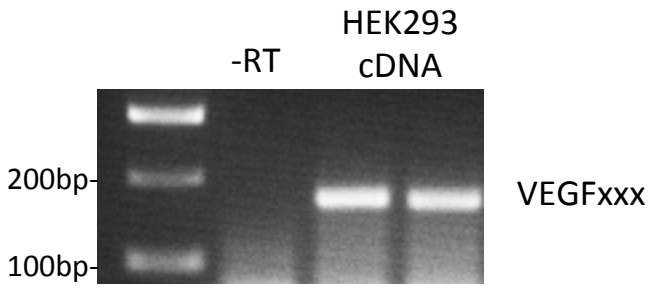


Figure 3-5 VEGF mRNA isoform expression in HEK293 cells
RNA was extracted from HEK293 cells and reverse transcribed. Primers that anneal with both $VEGF_{xxx}$ and $VEGF_{xxx}b$ were used to amplify the cDNA. Only pro-angiogenic $VEGF_{xxx}$ isoforms were detected (predicted size 175bp).

Reporter splicing can be easily visualised using fluorescent protein expression but has also been confirmed using the traditional RT-PCR method. Reporter specific primers were used to amplify transcripts produced from both proximal and distal splice site selection. The forward primer anneals to the artificial exon and reverse primer to the dsRED sequence (Figure 3-6). Proximally spliced mRNA would produce a PCR product of 193bp whereas a product of 128bp would be produced from distal splice site selection. As expected from the splicing pattern seen by fluorescence microscopy, only proximally spliced transcripts were detected, confirming that the reporter follows accurately the splicing pattern of the endogenous gene when stably-transfected in HEK293 cells.

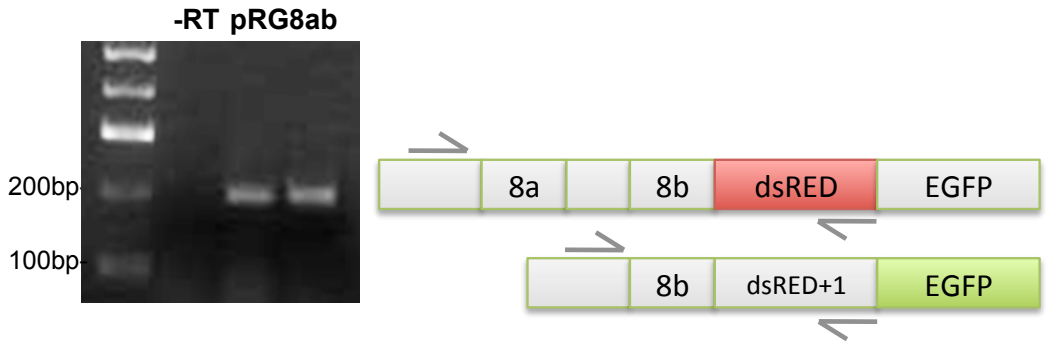


Figure 3-6 Reporter proximal splice site is predominantly used in HEK293 cells RNA extracted from reporter expressing HEK293 cells (pooled cells; stable transfections) was reverse transcribed; the cDNA was amplified using reporter specific primers. Only the PCR products corresponding to the proximally spliced pattern, ie 193bp in size, were detected.

The reporter was also transfected into other mammalian cell lines. A conditionally immortalized podocyte cell line (PCIP) has previously been shown to express high levels of anti-angiogenic $VEGF_{165}b$ (Amin *et al.*, 2011; Cui *et al.*, 2004). Electroporation was used to transiently transfect podocytes with pRG8ab and fluorescent micrographs taken of fixed cells. The podocytes expressed predominantly EGFP indicating anti-angiogenic style splicing via use of the distal splice site (Figure 3-7)

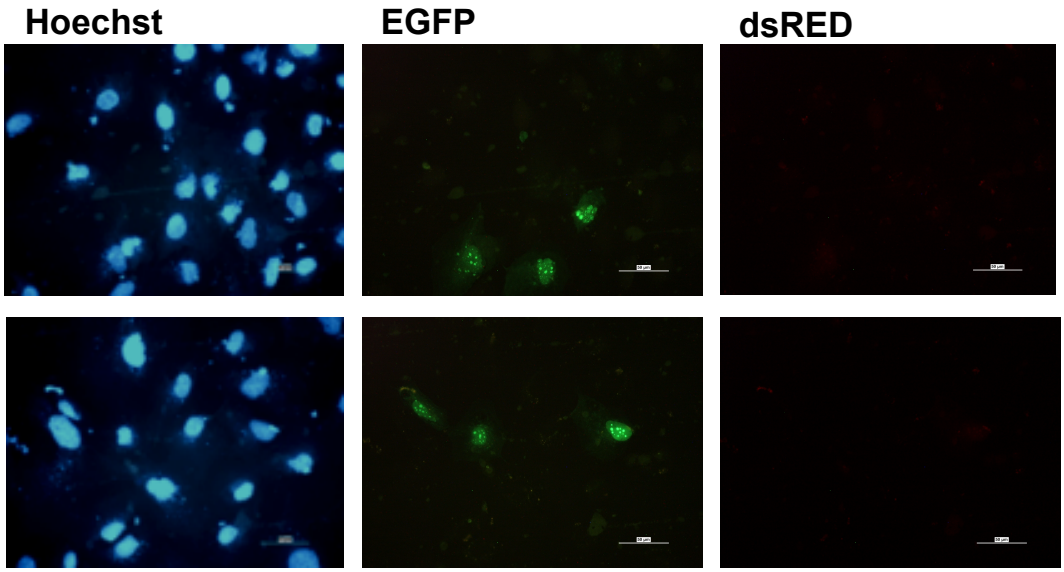


Figure 3-7 pRG8ab distal splice site is used during alternative splicing in proliferating conditionally immortalized podocytes
Selection of the VEGF reporter's distal splice site leads to EGFP expression. No dsRED, only EGFP expression was observed in transfected podocytes indicating anti-angiogenic splicing pattern.

A third cell line transfected with the reporter was PC3. It is recognised that anti-angiogenic VEGF isoforms are down regulated in prostate cancers compared to normal prostate tissue. Fluorescence from both EGFP and dsRED was observed when PC3 cells were transiently transfected with pRG8ab (Figure 3-8) indicating use of the proximal and distal splice sites of the reporter. Cells were cultured in 750ug/ml G418 to select for reporter expressing cells and create a stable cell line expressing the reporter plasmid. Similar to the effect seen in HEK293 cells, the PC3 stably transfected cells did not express EGFP, just dsRED. This suggests predominant use of the proximal splice site, correlating with the alternative splicing of the endogenous *VEGF* gene in these cells (Mavrou *et al*., 2014). These experiments suggest that the reporter is following accurately the splicing pattern of the endogenous gene in various cell lines.

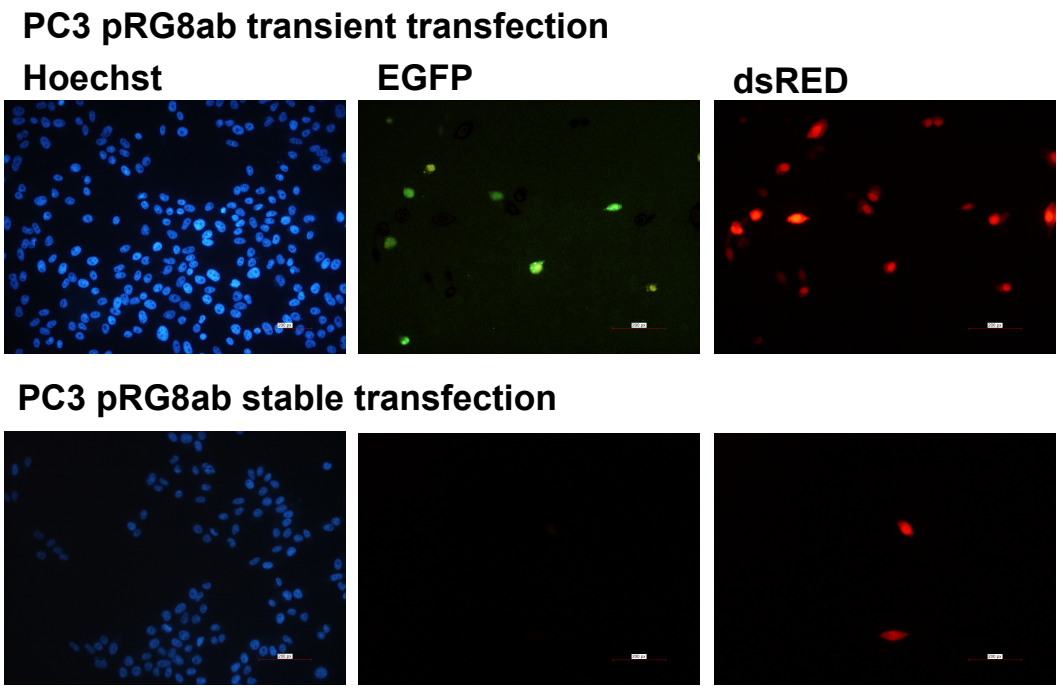


Figure 3-8 pRG8ab expression in the prostate cancer cell line, PC3
PC3 cells were transfected the pRG8ab and imaged. Transiently transfections expressed dsRED and EGFP. A PC3 pRG8ab stable cell line was created using antibiotic selection. Only dsRED expression could be detected in the stable transfections.

3.2.3 Fluorescence activated cell sorting of reporter cells

A main advantage of splicing reporters is that fluorescence can be used to quantify the splicing pattern within live cells. This can be achieved by using flow cytometry to measure the fluorescent protein expression within a sample of cells. Flow cytometry can quickly measure the fluorescence of a sample of millions of cells. Cells were collected for flow cytometry using trypsin to detach them from culture plates and resuspended in growth media containing 1% paraformaldehyde for fixation. For flow cytometric analysis, cells are transported through a fluidics system within the flow cytometer in single file where they are illuminated by a series of lasers. The size and granularity of cells is measured by the forward and side scatter caused when laser light hits a cell. A laser is used to excite fluorophores. In the HEK pRG8ab cells, both the fluorescent proteins were excited using a laser at 488nm wavelength. Different emission filters allowed detection of dsRED and EGFP and to count the number of cells expressing either or both proteins. The emission profile produced by HEK293 pRG8ab cells was compared to untransfected HEK293 cells (Figure 3-9). The emission produced by untransfected cells (background fluorescence) was used to create a gate (see left panel), any cells that produce fluorescence within this gate were expressing dsRED or EGFP. This was used to count the number of fluorescent cells within a sample.

After selection with geneticin, ~16% of the HEK293 cells population were expressing the reporter construct. Flow cytometry detected that the cells positive for reporter expression predominantly produced dsRED (Figure 3-9), right panel), indicating use of the proximal splice site. This corresponds with the splicing pattern seen via fluorescence microscopy and RT-PCR. For the purposes of further validation and screening a higher percentage of fluorescent cells was needed. The fluorescent population was increased by selecting cells using fluorescence activated cell sorting (FACS).

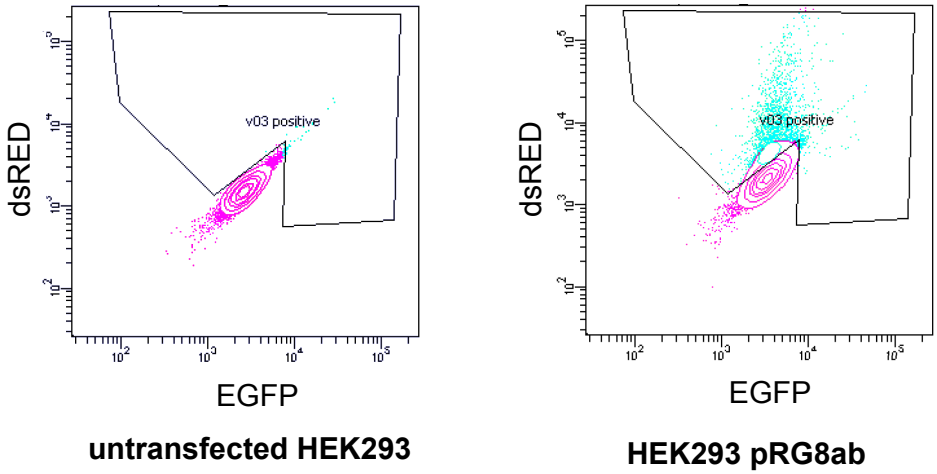


Figure 3-9 Flow cytometric analysis of reporter expressing cells
The fluorescent protein expression in reporter expressing HEK293 cells was measured by flow cytometry. Untransfected HEK293 cells were used as a negative control and to create a gate. Any cells which fell within this gate were regarded as transfected and therefore, expressing dsRED or EGFP.

Cell sorting allows different populations of cells to be isolated from each other. Different cell types can be separated by flow cytometry according to their size or fluorescently labelled on cell type-specific markers for separation via FACS. In this case, reporter-positive cells were able to be separated from the untransfected population as transfected cells expressed dsRED or EGFP producing a fluorescent signal. In the first instance, a sample of untransfected HEK293 cells were run through the cell sorter. Similarly as described for analytical flow cytometry, the scatter pattern produced by untransfected cells was used as a negative control. Using the sorter software, a gate was set around the scatter created by untransfected cells. When the pRG8ab transfected sample was analysed by the sorter, any cells that produced fluorescence (red or green) outside of the gate were deemed to be reporter positive (Figure 3-10).

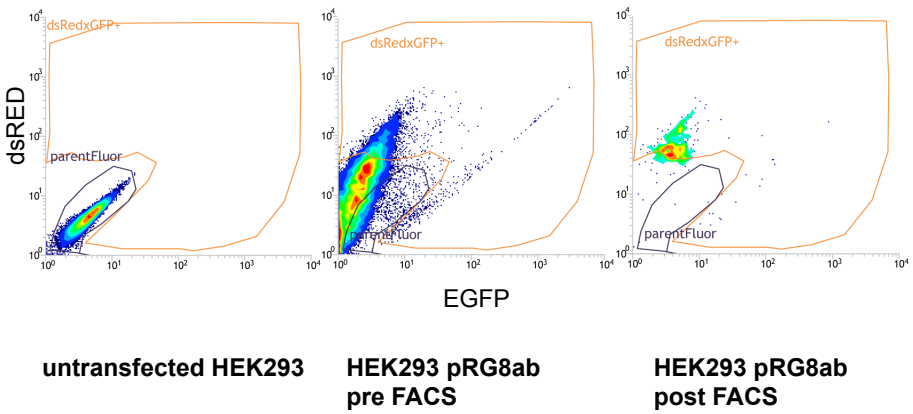


Figure 3-10 Fluorescent activated cell sorting of HEK293 pRG8ab cells
FACS was used to gain a higher percentage of fluorescent protein expressing cells within the HEK293 pRG8ab stably transfected cell line. Untransfected HEK293 cells were used as a negative control and to create gates that will measure and separate fluorescent cells from non-fluorescent cells.

Fluorescent, reporter-expressing cells were electrostatically separated from untransfected cells. Two rounds of FACS were performed to gain a stable HEK293 pRG8ab cell line in which 88% of the population were expressing the fluorescent reporter (Figure 3-11).

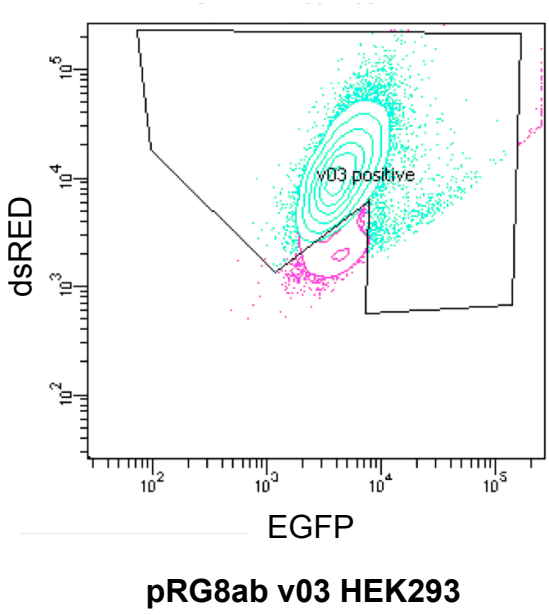


Figure 3-11 pRG8ab expression in HEK293 cells after fluorescence activated cell sorting
Cells were subjected to FACS to separate fluorescent, reporter expressing cells from non-fluorescent cells. After two rounds of FACS, 88% of the HEK293 pRG8ab population were fluorescent (v03 positive). dsRED was highly expressed (x axis) with only a small population expressing EGFP.

3.2.4 SRPK1 inhibition increases reporter distal splice site selection

Before using the VEGF fluorescent splicing reporter cells in a screening capacity, further validation was required to show that the reporter splicing accurately mimics endogenous *VEGF* splicing. The association between SRPK1, SRSF1 and exon 8 splicing regulation is well established (Gammons *et al*., 2014; Mavrou *et al*., 2014; Nowak *et al*., 2010; Oltean *et al*., 2012). Inhibition of SRPK1 reduces phosphorylation and activation of SRSF1. SRSF1 binds to *VEGF* mRNA and promotes selection of the proximal splice site of exon 8: SRPK1 inhibition reduces this interaction and increases the expression of anti-angiogenic VEGF proteins via increased use of the distal splice site. Therefore validation was attempted by inhibiting SRPK1 in reporter expressing cells. HEK293 pRG8ab cells were incubated with SRPIN340 and a series of newly developed SRPK1 inhibitors at 10μM for 48 hours. Effect of SPRK1 inhibition on reporter splicing within cells was observed using flow cytometry to measure expression of dsRED (pro-

angiogenic splicing) and EGFP (anti-angiogenic splicing) compared to control treatment

As described previously, gates were created within the flow cytometry software to measure the percentage of cells that express dsRED or EGFP, which was used to indicate which splice site is being selected. The percentage of cells with a sample that express dsRED or EGFP were used to calculate the ratio of splice site selection. Changes in dsRED/EGFP ratio implied altered splice site selection. The sum of the two population percentages (dsRED+EGFP) was calculated and used as an indicator of increases or decreases in the overall expression of the reporter construct; this may be caused by effects of transcription, translation or fluorescent protein stability. Treatment with SRPIN340 did not cause a change in reporter splicing at 10μM. Two of the other SRPK1 inhibitors used, SPHINX and SPHINX7, have greater potency than SRPIN340 shown in an in vitro kinase assay (Gammons *et al*., 2013a). SRPIN340 and SPHINX produced a slight decrease in the ratio but it was not statistically significant. Treatment with SPHINX7 caused a significant decrease in dsRED/EGFP i.e increased use of the reporter distal splice site. Additionally, cells were treated with a negative control compound (AZ2.46) that had a similar molecular structure to the inhibitor SPHINX7 but does not block SRPK1 activity. AZ2.46 treatment did not cause a change from the DMSO control (Figure 3-12). This further demonstrated that the VEGF reporter splicing responds to stimuli in a similar way to endogenous *VEGF* mRNA and therefore would be a useful tool for investigating exon 8 alternative splicing.

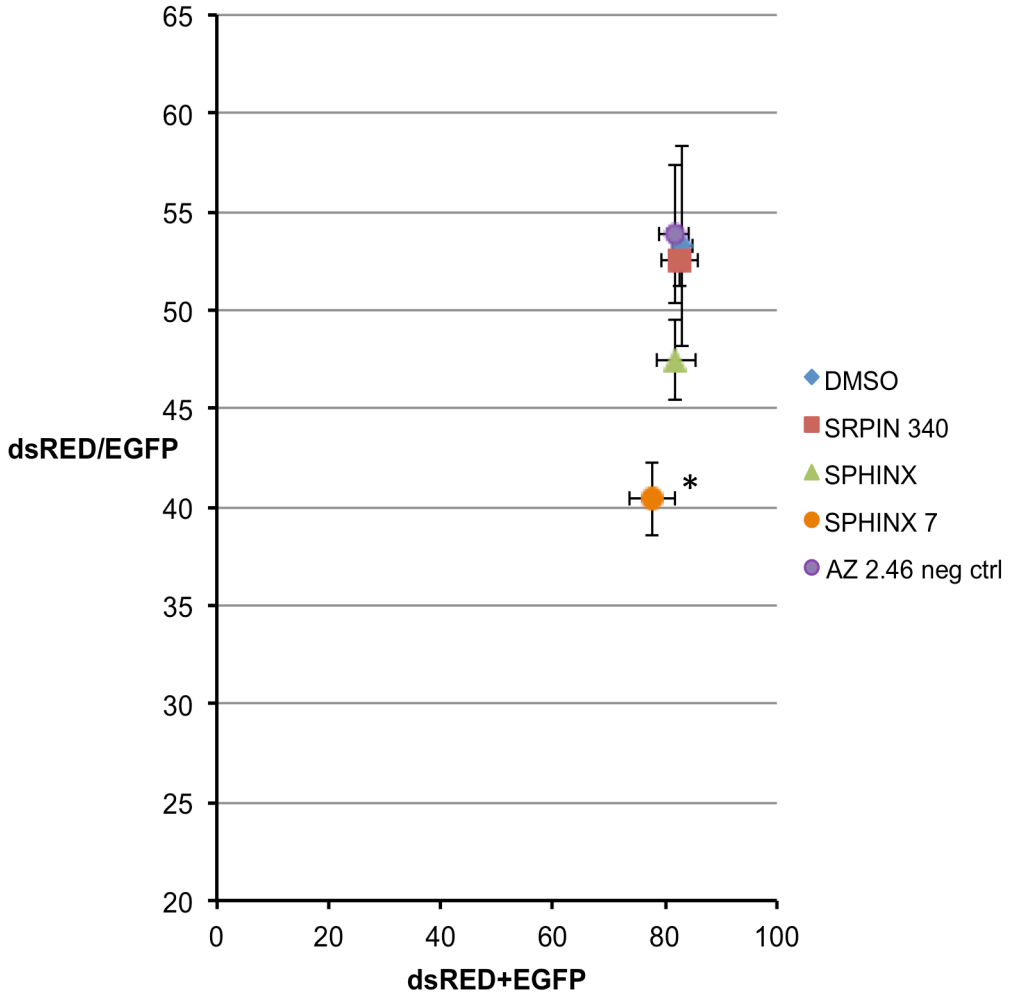


Figure 3-12 SRPK1 inhibition increases use of the pRG8ab reporter distal splice site
*HEK293 pRG8ab cells were treated with SRPK1 inhibitors at 10µM for 48 hours. After treatment, live cells were collected and flow cytometry used to measure the percentage of cells expressing dsRED and EGFP. Percentages were used to calculate the ratio of splice site selection (dsRED/EGFP). A decrease in the ratio suggests increased selection of the distal splice site. Changes in overall reporter expression are indicated by dsRED+EGFP. The effect of treatment on alternative splicing are measured by dsRED/EGFP. n=3, *P<0.05. No significant difference in RFP+GFP measurements.*

3.2.5 *Preliminary screens using pRG8ab to identify novel regulators of VEGF alternative*

As discussed, splicing reporters are a useful tool to identify novel regulators of an individual alternative splicing event. This may be achieved via overexpression or silencing of different genes, stimulation with signalling molecules or using small molecules inhibitors/agonists. Such reporters can be used in large high-throughput scale screens, but should be first be trialled in preliminary experiments.

It is recognised that growth factors can modify alternative splicing patterns by activation of cell signalling pathways (Blaustein *et al*., 2005; Matter *et al*., 2002; Pelisch *et al*., 2005; Zhou *et al*., 2012). IGF1, TNFα and TGFβ1 have all previously been shown to influence *VEGF* exon 8 alternative splicing (Nowak *et al*., 2008). A small group of growth factors were tested for their effect on pRG8ab alternative splicing (Figure 3-13). HEK293 pRG8ab cells were treated with each growth factor at 100nM for 48hours and flow cytometry used to quantify the alternative splicing of pRG8ab. EGF and FGF2 displayed the greatest effect on reporter splicing. Both increased the use of the distal splice site seen by increased EGFP expression. The other growth factors did not cause any considerable change to reporter splicing.

Additionally, a small cohort of small molecule inhibitors were used treat HEK293 cells and activity on pRG8ab alternative splicing examined (Figure 3-14). 5-Aza-2'-deoxycytidine (5'AZAdC), BIX02194 trihydrochloride hydrate and 3-deazaneplanocin A (DZnep) are all compounds known to cause changes to epigenetic marks. 5'AZAdc is an inhibitor of DNA methylation, BIX02194 is an inhibitor of histone-lysine methyl transferases, and DZnep can block histone 3 lysine 27 and histone 4 lysine 20 trimethylation. HEK293 pRG8ab cells were treated with 10µM of each epigenetic modifier for 48 hours. Following treatment, the cells were collected for analysis of reporter alternative splicing patterns via flow cytometry. 5'AZAdC did not have an effect on the dsRED/EGFP

expression ratio of the cells, however, BIX02194 significantly increased and DZnep significantly decreased the ratio, without marked changes to sum of dsRED and EGFP expression, indicating these drugs may change the pattern of reporter alternative splicing. A previous study has demonstrated DZnep to exhibit anti-angiogenic activity (Smits et al., 2011; Smits et al., 2010).

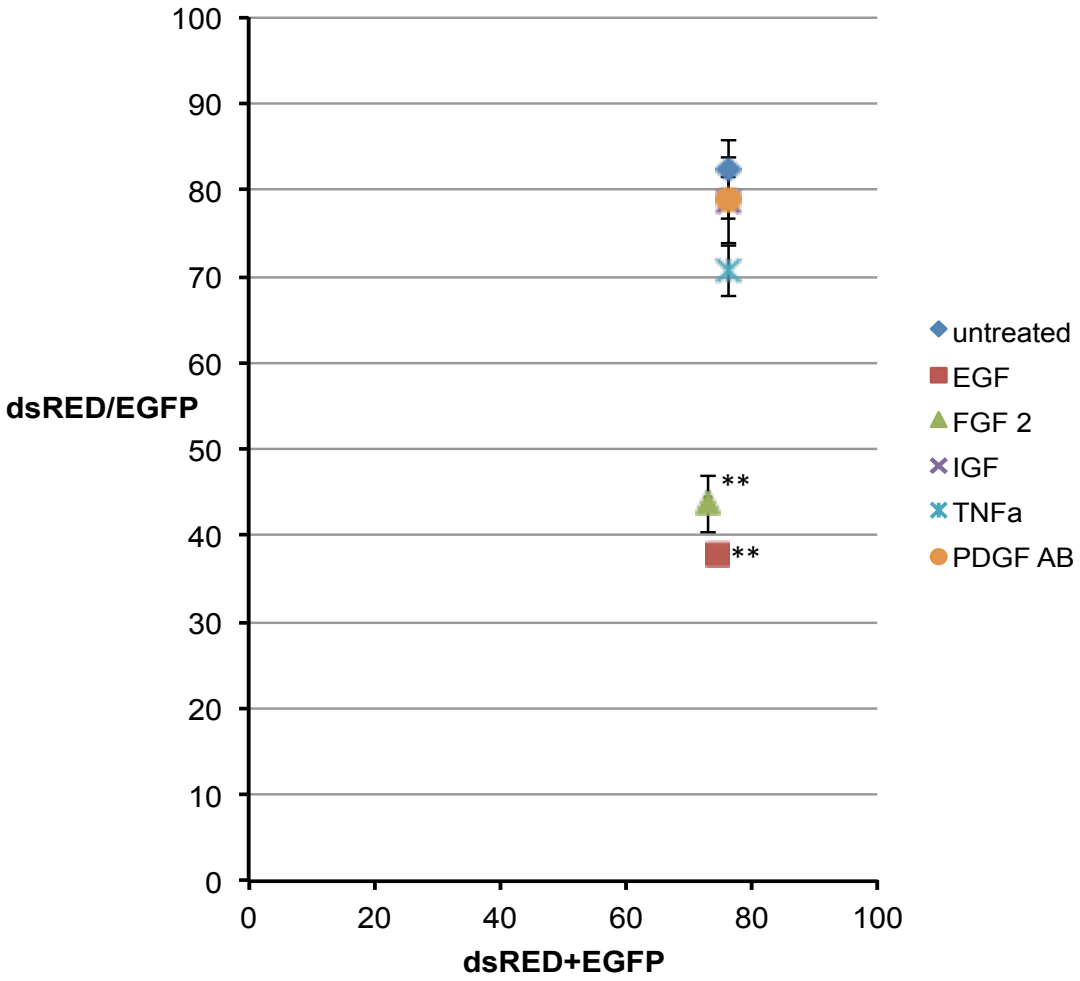


*Figure 3-13 The effect of a growth factor panel on reporter alternative splicing HEK293 pRG8ab cells were treated with 100nM EGF, FGF2, IGF1, TNFα or PDGF for 48 hours. The treated cells were collected and reporter alternative splicing was quantified using flow cytometry to measure dsRED and EGFP expression. n=3, **P<0.01. FGF2 also significantly changed dsRED+EGFP.*

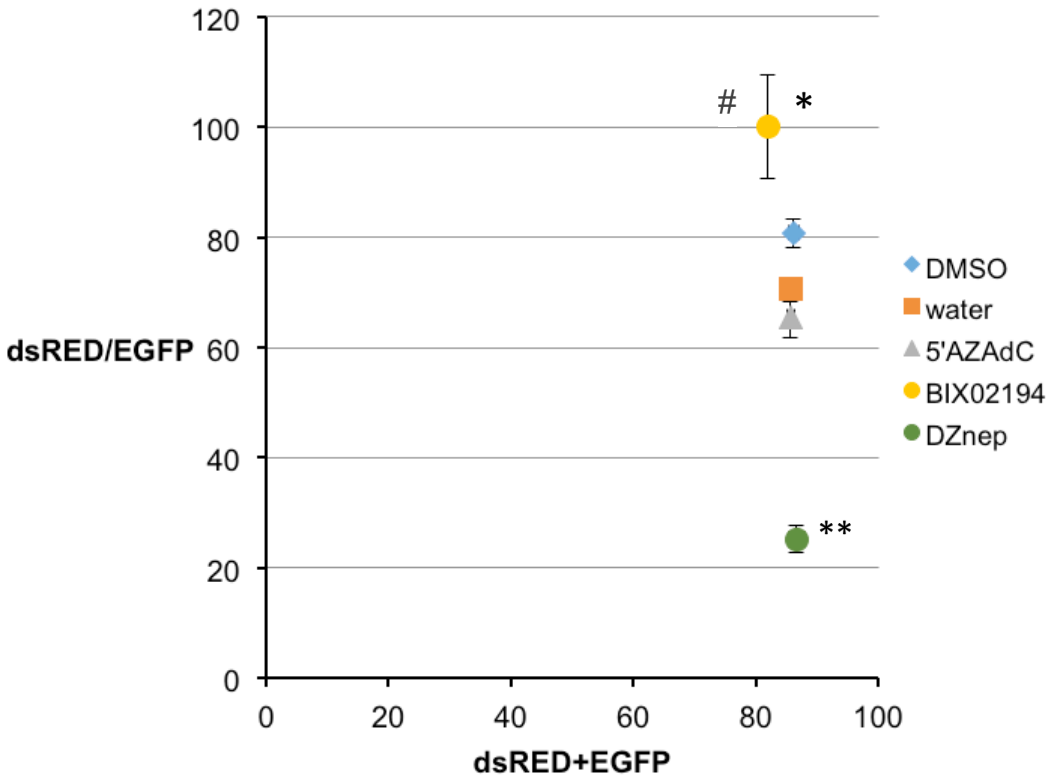


Figure 3-14 The effect of epigenetic modifying compounds on VEGF SSFR alternative splicing.
*HEK293 cells were treated with 10µM 5'AZAdC, BIX02194 or DZnep for 48 hours. Subsequently, dsRED and EGFP were measured by flow cytometry. dsRED/EGFP changes indicated a change in the ratio of reporter splice site selection. Total expression of the reporter was indicated by dsRED+EGFP. DZnep treatment is compared to water control. DMSO was used a control treatment for BIX02194 and 5'AZAdc. n=6, *P<0.05 vs. DMSO dsRED/EGFP, **P<0.01 vs. water dsRED/EGFP. $^{\#}$P<0.01 vs. DMSO dsRED+EGFP.*

Increasingly, naturally occuring compounds, such as resveratrol, are being investigated to elucidate potential benefits and possible exploitation for medical use. DIAVIT is a commercially available natural extract from bilberries and buckthorn. It has been used to alleviate complications of diabetes, including diabetic retinopathy, which is characterised by microvascular changes in the eye. Reporter-expressing HEK293 cells were cultured in three dilutions of DIAVIT. One tablet of DIAVIT was dissolved in 100ml of distilled water and filtered using a 0.2µm filter. This dilution was termed 'high dose' and 100µl was added to cells in 6-well plates. A further 10x and 100x dilutions were termed mid and low dose,

respectively. Following 48 hours of DIAVIT treatment, cells were collected and flow cytometry used to measure dsRED and EGFP-positive cells. DIAVIT treatment resulted in a dose-dependent decrease in dsRED/EGFP, suggesting increased use of SSFR distal splice site – this may provide a mechanistic explanation for the anti-angiogenic effect seen in patients with diabetic retinopathy that take DIAVIT as a adjuvant therapy. DIAVIT is marketed as a diabetic therapy, however, there are no published trials demonstrating these effects. The total expression of the reporter was was increased, as measured by the total number of cells expressing either of the fluorescent proteins (Figure 3-15)

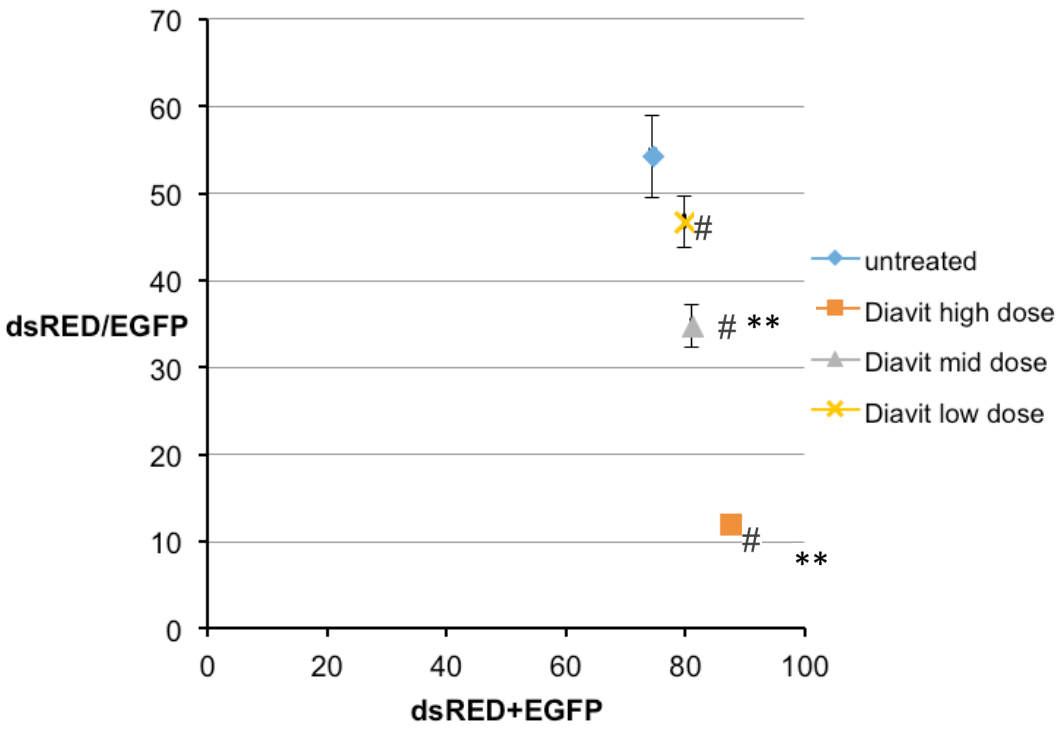


Figure 3-15 Natural extract DIAVIT reduces the ratio of VEGF SSFR splice site selection
*HEK293 cells were cultured with three increasing doses of a DIAVIT solution for 48 hours. n=3, **P<0.01 vs. untreated dsRED/EGFP. #P<0.01 vs. untreated dsRED+EGFP.*

3.3 DISCUSSION

3.3.1 VEGF splicing-sensitive fluorescent reporter is expressed and follows endogenous VEGF splicing patterns in vitro

Signalling stimulated by VEGF is crucial in the process of angiogenesis and has been intensively studied in physiology and pathology (Carmeliet, 2003; Carmeliet and Jain, 2000; Ferrara *et al*., 2003). Discovering a family of alternatively spliced VEGF protein isoforms with anti-angiogenic properties added a new layer of complexity to the role of VEGF (Bates *et al*., 2002; Bates and Harper, 2005). Pro-angiogenic $VEGF_{xxx}$ is upregulated in tumours whereas $VEGF_{xxx}b$ isoforms are decreased (Amin *et al*., 2011; Pritchard-Jones *et al*., 2007). It is therefore important to understand what controls the balance between pro and anti-angiogenic VEGF.

The VEGF pro/anti-angiogenic splicing reporter, pRG8ab, was designed to investigate the mechanisms that regulate exon 8 3' splice site selection and find novel compounds that can modify the splicing decision. The design was adapted from the RG6 bichromatic splicing reporter developed by Orengo *et al.* Before use in a screening scenario, the reporter first had to be validated to confirm if it would be useful to find new regulators of pro vs. anti-angiogenic *VEGF* splicing. It needed to be ascertained if the reporter construct accurately mimicked the alternative splicing of *VEGF* mRNA. For validation, pRG8ab was transfected and expressed in several cell lines and known regulators of *VEGF* exon 8 alternative splicing used to alter pRG8ab splicing.

It was first determined whether the pRG8ab plasmid was well expressed when transfected into mammalian cell lines. Transient transfection was first attempted in HEK293 cells using lipofection to introduce the plasmid into the cells. Forty-eight hours after transfection, expression of both dsRED and EGFP could be detected in cells (Figure 3-3). This confirmed expression of the plasmid in culture and that both reporter splice sites, proximal and distal, can be selected and produce fluorescent protein. The gene expression produced by transient

transfection is not permanent. As the cells replicate and divide the plasmid may be lost, therefore stable transfections were made. In these cells, EGFP expression could no longer be seen when imaging the cells, whereas dsRED was highly expressed indicating proximal splice site selection (Figure 3-4). This corresponds to the alternative splicing of *VEGF* in this cell line, the proximal splice site is dominant causing pro-angiogenic splice site selection. The change in the splicing pattern between transient and stable transfections may be an issue caused by copy number. After transient transfection, there are likely to be many copies of the plasmid in each transfected cell and being highly expressed. Under these circumstances, the splicing machinery may not act in its usual manner and the splice factors needed at a certain splice site diluted out resulting in an artificial splice pattern (Cooper, 2005; Libri *et al*., 1989). In the HEK293 pRG8ab stably transfected cells, the plasmid has been integrated into the genome of the cells and copy number should be lower. Here, the reporter splicing seems to be closer to the endogenous pattern. A similar effect was observed when PC3 cells were transfected (Figure 3-8). The predominant use of the proximal 5' splice site of the reporter in stable HEK293 pRG8ab cells was confirmed by RTPCR and flow cytometry.

Another of the cell lines transfected with pRG8ab was PCIP (proliferating conditionally immortalized podocytes). Anti-angiogenic VEGF isoforms are highly expressed in this cell line (Cui *et al*., 2004). Podocytes were transiently transfected with pRG8ab using electroporation. Fluorescence microscopy showed the transiently transfected PCIP cells expressed only EGFP and no dsRED, indicating the distal splice site of the reporter is being predominantly selected. This mimics with the splicing of *VEGF* mRNA in this cell type but contrast with the situation seen when PC3 and HEK293 cells were transiently transfected; both dsRED and EGFP were highly expressed in transients. This may be caused by the use of two different methods to transfect with cells; lipofection was used to express the pRG8ab plasmid in PC3 and HEK293 cells whereas podocytes were transfected using electroporation. The transfection efficiency of the reporter into podocytes was relatively low compared to the other cell lines, this may mean

that less copies of the plasmid were integrated in each cell. Less integrants would not have such a disruptive effect on the splicing machinery leading to the reporter transcripts to be alternatively spliced in the same pattern as the endogenous *VEGF* mRNA.

3.3.2 Inhibition of SRPK1 modifies reporter alternative splicing

In order to maximise the potential of reporter expressing cells for screening and measuring changes in reporter splicing, it was necessary to have a high percentage of the cells expressing the fluorescent proteins. Fluorescent cells were isolated using FACS to obtain a population in which 88% of the cells are expressing dsRED or EGFP. SRPK1 and the downstream splice factor SRSF1 are known to be important for promoting the selection of *VEGF* exon 8 proximal splice site and therefore producing pro-angiogenic VEGF isoforms. Knockdown or chemical inhibition of SRPK1 reduces the expression of $VEGF_{165}$ and increases $VEGF_{165}b$ production. SRPIN340 and other selective SRPK1 inhibitors (SPHINXes) have been shown to be anti-angiogenic in vitro and in several *in vivo* models (Gammons *et al*., 2013a; Gammons *et al*., 2013b). SRPIN 340 did not have an effect on pRG8ab splicing but SPHINX caused a slight decrease in dsRED/EGFP ratio. A reduction in dsRED/EGFP suggests SRPK1 inhibition has increased distal splice site selection and reduced use of the proximal splice site. SRPIN 340 and SPHINX have similar IC_{50} values for inhibiting SRPK1. SRPIN340 has inhibitory activity on SRPK1 and SRPK2 whereas SPHINX is more specific for SRPK1. The inhibitor that causes the most potent SRPK1 inhibition, SPHINX7, had the greatest effect on reporter splicing. A negative control compound, AZ2.46, was also used to treat reporter cells and caused no change to reporter alternative splicing. AZ2.46 has a similar molecular structure to the SRPK inhibitors but does not actively inhibit SPRK activity.

3.3.3 Pilot screens using pRG8ab to measure alternative splicing switches

Upstream of splice factors and kinases, growth factors can influence cell signalling to cause changes in alternative splicing. Some pathways have previously described the effect of certain growth factors on *VEGF* terminal exon alternative splicing (Nowak *et al*., 2008). IGF1 and TNFα were shown to cause preferential selection of the proximal splice site whereas distal splice site selection was induced by TGFβ. EGF has also been implicated in alternative splicing regulation via signalling to Akt, which causes SRPK1 and 2 activation (Zhou *et al*., 2012). Reporter expressing HEK293 cells were treated with a series of growth factor and their alternative splicing measured using fluorescent protein expression. IGF1 and TNFα did not alter reporter splicing. Under basal conditions in this cell line, reporter proximal splice site selection is predominant shown by high dsRED expression, therefore if PSS selection is already at a maximum IGF and TNFα many not have a measurable effect on reporter splicing. EGF and FGF2 both induced a decrease in the RFP/GFP ratio indicating increased use of the anti-angiogenic distal splice site. This is despite EGF being known to stimulate SRPKs which induce PSS use via SRSF1. This effect may be due to the other splice factors that are influenced by EGF stimulation. The effect of EGF on SRPKs is thought to be dependent on Akt, but Akt also activates several other splice factors kinases including Clk (Jiang *et al*., 2009; Li *et al*., 2013). One of the splice factors phosphorylated by Clk is SRp55 (also known as SRSF6), which increases $VEGF_{xxx}b$ expression through increased DSS selection (Nowak *et al*., 2008). Therefore it may be activation of Clk caused by EGFR signalling that creates increased EGFP expression from the reporter construct.

Another of the pilot screens conducted used a series of small molecules known to interfere with epigenetic modifications. The association between epigenetic marks and alternative splicing is a relatively new area of research but is rapidly evolving. It is now understood that chromatin organisation, DNA methylation and histone modifications may all impact alternative splicing regulation.

Mapping of the position of nucleosomes within the whole genome has revealed the boundaries where introns and exons meet to be enriched with nucleosomes, potentially indicating nucleosomes to have a role in exon definition and alternative splicing decisions (Andersson *et al*., 2009). Nucleosome mapping also demonstrated that excluded exons are less enriched in nucleosomes than exons included in spliced transcripts (Schwartz *et al.*, 2009).

Chromatin conformation is a major mechanism used to control gene expression, with transcription blocked when chromatin is in 'closed' conformation. Chromatin remodellers, including methyltransferases, change the conformation to enable or inhibit transcription and to effect alternative splicing events. Certain methyltransferases have been demonstrated to interact with snRNPs (Cheng et al., 2007; Martinez et al., 2001). The SWI/SNF nucleosome-remodelling complex has also been shown to mediate changes to alternative splicing via association with and recruitment of the U1 and U5 snRNPs (Batsche et al., 2006).

Histone modifications are known to mediate changes in gene expression by changing chromatin structural conformation and recruitment of other factors. Their role as regulators of alternative splicing is an emerging area of investigation. Genome mapping of various common histone modifications has demonstrated some histone marks to be enriched or depleted in exons compared to the surrounding introns (Schwartz et al., 2009). Additionally, the histone deacetylase inhibitor, trichostatin A, was shown to promote skipping of specific exons in fibronectin and neural cell adhesion molecule mRNA (Nogues et al., 2002; Schor et al., 2009).

The FGFRII mRNA contains two mutually exclusive exons, IIIb and IIIc. Inclusion of either alternative exon in the final transcript is highly cell type-specific, with IIIb being maintained in epithelial cells and IIIc in mesenchymal cell types. Investigation demonstrated the gene to be enriched in different histone modifications depending on which exon is normally spliced. H3K27me3 and H3K4me3 marks were enriched in IIIb expressing cells, and H3K27me3 and

H3K4me1 were present in cells that include IIIc. Overexpression or RNAi knockdown of the required histone methyltransferases was able to modify FGFRII alternative splicing to promote or inhibit IIIb or IIIc splicing (Luco et al., 2010). Collectively, these observations indicate that modulation of epigenetic marks and changes to chromatin organisation can alter splicing decisions.

Of the three epigenetic-modifying small molecules tested, one increased and one decreased dsRED/EGFP expression ratio of the VEGF SSFR. DZnep induced a significant decrease in the dsRED/EGFP indicating increased use of the reporter distal splice site, the splice site used to create anti-angiogenic VEGF isoforms from the endogenous *VEGF* mRNA. DZnep inhibits trimethylation of histone 3 lysine 27 and histone 4 lysine 20. The small molecule acts via an indirect inhibitory mechanism. DZnep inhibits the enzyme *S*-adenosylhomocysteine hydrolase, which metabolises *S*-adenosylhomocysteine to adenosine and homocysteine. Increased concentration of *S*-adenosylhomocysteine caused by this results in inhibition of reactions that are *S*-adenosylmethionine dependent including histone methylation, in which methyltransferases use *S*-adenosylmethionine as a methyl donor. Trimethylation of H3K27 by EZH2, a methyltransferase component of the Polycomb repressor complex (PRC), is inhibited by DZnep. H3K27me3 is associated with repressive chromatin conformation and EZH2 overexpression has been observed in several types of cancer. Through inhibition of these mechanisms, DZnep has demonstrated the ability to re-express genes switched off in cancer and stimulate apoptosis of cancer cells (Sha *et al.*, 2015).

When used to treat HEK293 cells expressing the VEGF SSFR, DZnep produced a marked decrease in the ratio of dsRED/EGFP expression. This indicated that DZnep reduced use of the reporter's proximal splice site, while increasing distal splice site selection. DZnep, or other histone-modifying compounds have not been previously demonstrated to modify *VEGF* alternative splicing. However, DZnep or knockdown of EZH2 have been shown to effect angiogenesis and VEGF expression. DZnep inhibition of the PRC was shown to decrease VEGF levels in

endothelial cells. Smits *et al* used DZnep to reduce proliferation, migration and invasion of glioblastoma cells *in vitro*. Gene ontology analysis found that expression of 33 genes associated with angiogenesis were correlated with expression of EZH2 in glioblastoma samples. Additionally, the study co-cultured endothelial cells with glioblastoma cells that had been pre-treated with DZnep or EZH2 siRNA. The cells were cultured on Matrigel and the effect on tubule length and branching measured. Inhibition of EZH2 by siRNA or DZnep reduced endothelial tubule formation. Furthermore, the effect of EZH2 on tumour growth was investigated further *in vivo*. Glioblastoma cells were subcutaneously injected into nude mice and tumour growth monitored. Tumour growth was reduced by intravenous treatment with DZnep (Smits et al., 2010). DZnep treatment induced a marked change to the ratio of VEGF SSFR splice site selection. Use of the distal splice site, which is used to produce anti-angiogenic VEGF isoforms from the endogenous mRNA, was significantly increased. Another of the small molecules used in the small pilot screens was BIX02194, a selective inhibitor of G9a histone methyltransferase. BIX02194 significantly increased the ratio of dsRED/EGFP positive cells compared with DMSO control-treated cells. The association between histone modifications and *VEGF* alternative splicing, and how this may be involved in the regulation of angiogenesis, is an area that requires further investigation.

Using naturally occurring compounds as therapeutic agents is an attractive prospect as they are often readily available and may not have extreme side effects or toxicity. DIAVIT, a natural extract from bilberry and sea buckthorn, was tested for its effect on the alternative splicing of the VEGF SSFR. In HEK293 cells, DIAVIT significantly increased the number of cells that expressed EGFP indicating increased selection of the reporter distal splice site. As DIAVIT is a natural extract, it is comprised of numerous different compounds. Any one, or a combination of several, may cause the alternative splicing change. DIAVIT contains many anthocyanins, a class of flavonoid molecules. Anthocyanins are found in many fruits, vegetables and other natural products, and have been observed to protect against various diseases. The reported biological effects of

anthocyanins include inhibition of certain enzymes, increased cytokine expression, reduced permeability of microvessels and anti-inflammatory action (Lila, 2004). Anthocyanins and other flavonoids are being studied to elucidate the mechanisms by which they regulate physiological functions. DIAVIT is marketed as a diabetic therapy, however, there are no published trials demonstrating these effects. Further investigation has demonstrated DIAVIT to be protective in a mouse model of diabetes (Stevens, unpublished) and more research is required to isolate the active compounds within the extract that cause such effects.

In conclusion, a splicing-sensitive fluorescent reporter was designed to investigate the alternative splicing decision that creates two families of VEGF isoforms with either pro- or anti-angiogenic properties. The reporter was validated for use by transfection into different cell lines and treatment with small molecule inhibitors targeting pathways known to influence *VEGF* splicing decisions. This enabled the reporter to be approved for use in larger unbiased screens attempting to find chemicals that can alter *VEGF* alternative splicing and change the balance between pro- and anti-angiogenic isoforms.

Chapter 4

Screening for small molecules that can modulate *VEGF* alternative splicing

4.1 INTRODUCTION

Angiogenesis is required for the growth of solid tumours. Nutrients and oxygen are supplied to the tumour and waste products removed (Bergers and Benjamin, 2003). The development of new vessels through angiogenesis predominantly depends on VEGF. Endothelial cells express VEGFR-2, which initiates angiogenic signalling pathways upon VEGF binding. VEGF expression is increased in many tumours- secreted from cancer cells and associated stromal cells (Dvorak et al., 1995; Gerber et al., 1999).

There has been an abundance of investigations into finding anti-angiogenic drugs for use in the treatment of cancers, with the majority targeting VEGF or VEGFRs. A humanized monoclonal anti-VEGF antibody, bevacizumab, gave patients with metastatic colorectal cancer improved survival when combined with chemotherapy in a randomised phase three clinical trial (Hurwitz et al., 2004)and has also shown use in treating advanced non-small cell lung cancer (Hurwitz et al., 2004; Sandler et al., 2006) . Other anti-angiogenic agents that have been trialled include the small molecule receptor tyrosine kinase inhibitors (RTKIs), sorafenib and sunitinib, which inhibit several RTKs including VEGF receptor-2 (Escudier et al., 2007; Motzer et al., 2007). But so far, most angiogenesis targeting cancer treatments have only provided moderate benefits and are associated with considerable side effects (Eskens and Verweij, 2006).

The anti-angiogenic $VEGF_{xxx}b$ isoforms are produced by alternative splicing of *VEGF* mRNA. The proteins produced only differ in the six amino acids at the carboxy terminal.(Bates et al., 2002) $VEGF_{xxx}b$ makes up a major proportion of total VEGF expression in many normal tissues and is decreased in tumours. Use

of a plasmid to overexpress $VEGF_{165}b$ slows the growth of tumours in kidney, colon and prostate models (Rennel et al., 2008; Woolard et al., 2004).

Even since the discovery of anti-angiogenic VEGF isoforms, most studies investigating VEGF and anti-VEGF agents have not distinguished between the two isoform families. A study has shown that bevacizumab inhibited the quick growth of tumours expressing $VEGF_{165}$ in mice, but was not as effective at inhibiting the slower growth of tumours expressing $VEGF_{xxx}b$. Considering this, it is important to develop anti-angiogenic agents that specifically target $VEGF_{xxx}$ or to alter *VEGF* splicing, reducing the pro-angiogenic and enhancing anti-angiogenic VEGF expression (Varey et al., 2008b).

Alternative splicing is a fundamentally important step of gene regulation. Thus, much investigation has focused on how differential splicing is regulated and how this goes awry during disease. One technique to study an individual splicing event is high throughput screening, in which many siRNAs, cDNAs or chemicals can be assayed for their effect on the alternative splicing of a gene or construct. This can give insight into alternative splicing control and potentially find chemicals that can be used as a therapeutic in diseases that have altered splicing of the target transcript.

Splicing reporters have been successfully used in siRNA, cDNA and small molecule screens to investigate particular alternative splicing events (Naryshkin et al., 2014; Stoilov et al., 2008; Warzecha et al., 2009). Screening for small molecules that modulate *VEGF* alternative splicing could give more insight into which pathways are involved in regulating this splicing event and how it is altered in cancer. This could provide potential new anti-angiogenic therapeutics. Using the VEGF reporter we aimed to screen for small molecules that could increase use of *VEGF*'s distal exon 8 splice site and/or reduce proximal splice site selection.

4.2 RESULTS

4.2.1 A primary screen using the Library of Pharmacologically Active Compounds

In PC3 cells transfected with the pRG8ab construct, reporter pre-mRNA is predominantly spliced at the proximal 5' splice site, resulting in high levels of dsRED expression. This correlates with the alternative splicing pattern of the endogenous *VEGF* gene- PC3 cells highly express the pro-angiogenic VEGF isoforms. A small molecule screen was performed to identify compounds that can alter VEGF reporter alternative splicing.

The screen was performed using the VICTOR X multi-label plate reader. The plate reader has a variety of parameters that can be altered to achieve the most sensitive fluorescent detection. This includes different filters, excitation lamp energy, size of the measurement aperture etc. Combinations of different parameters were tested to optimise detection of increasing/decreasing levels of dsRED and EGFP expression. The PC3 pRG8ab cell line was used to optimise dsRED measurement as the cells predominantly produce red fluorescence due to selection of the reporter proximal splice site. A second cell line, with a different reporter construct, was used for EGFP detection. HEK293 cells were transfected with a fluorescent reporter based on the alternative splicing of FGFRII, in these cells EGFP is highly expressed (Oltean et al., 2006). Decreasing number (50,000 to 5000) of each cell type were mixed with non-fluorescent cells and seeded into 96 well plates and different parameter combinations were used to measure the levels of each fluorescent protein. Excitation and emission filters with narrow bandwidth ranges were used to avoid bleed-through between the two fluorescent channels when detecting each protein. Measurements were plotted and the R^2 value for each line calculated to determine which parameter combination that most accurately reflected the linear decrease in cell number/fluorescence (Figure 4-1). The chosen parameters for fluorescent detection are summarised in Table 3.

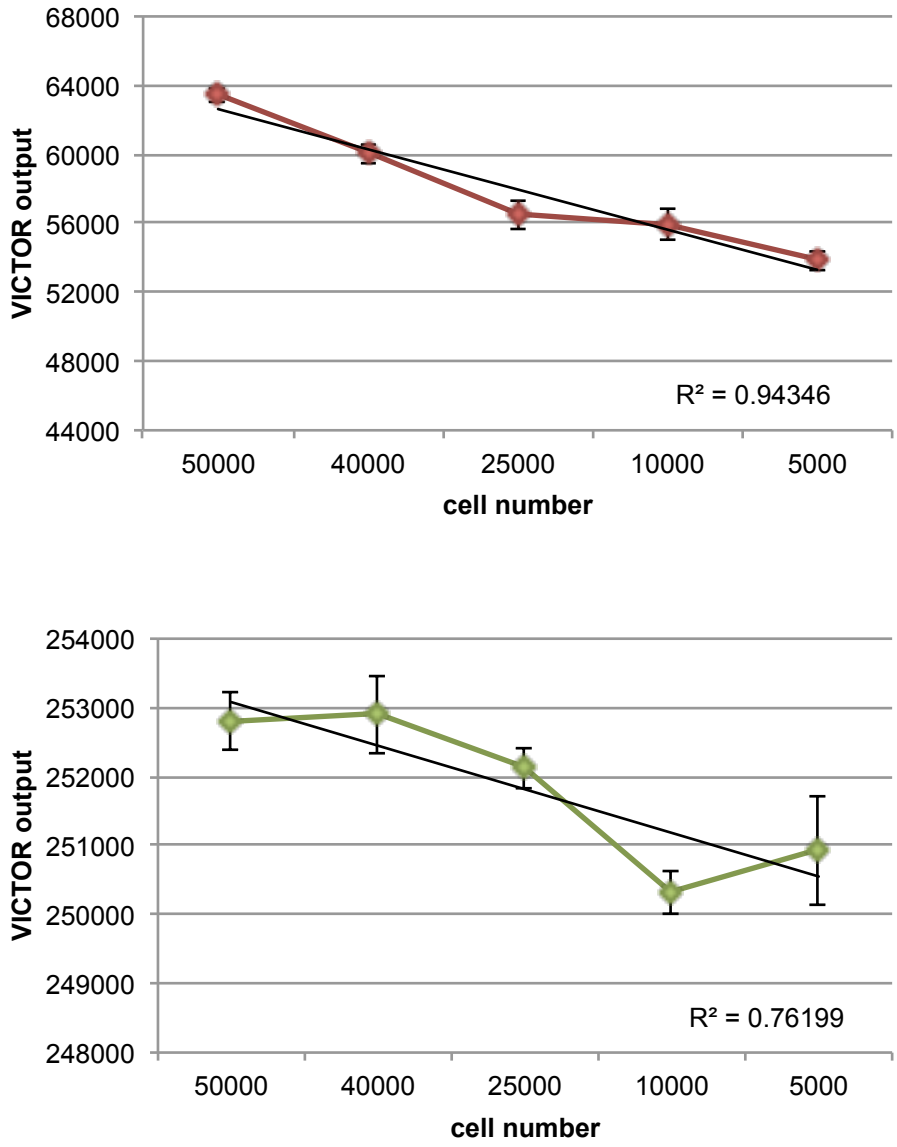


Figure 4-1 Optimised conditions for detecting fluorescent proteins using VICTOR plate reader and pRG8ab VEGF reporter cells.
(A) dsRED was measured using excitation filter with excitation range at 550 +/- 10nm and emission filter that detects at 632 +/- 10nm. (B) EGFP was measured using excitation filter with excitation range at 485 +/- 10nm and emission filter that detects at 535 +/- 10nm.

	dsRED	EGFP
Excitation filter (nm)	550	485
Emission filter (nm)	632	535
Measurement height (mm)	3 (above)	
Measurement time (secs)	3	
Emission aperture	Small	
Lamp energy	30000	

Table 3 Fluorescent protein measurement parameters used during chemical screen.
Each of the excitation or emission filters used were of the narrow bandwidth variety, only allowing passage of light within a +/- 10nm range of the stated wavelength.

The chemical library that was chosen to be used during the screen was LOPAC®,[1280] produced by Sigma-Aldrich. The acronym LOPAC stands for Library of Pharmacologically Active Compounds. All the 1280 compounds of the LOPAC have known pharmacological activity and had previously been approved for use by the U.S. Food and Drug Administration. This is a broad-spectrum library, which contains molecules that would target many major receptor and enzyme classes such as GPCRs, ion channels and kinases.

A primary screen was performed to identify compounds that can alter reporter alternative splicing in PC3 cells (Figure 4-2, step 1). 10,000 PC3 pRG8ab cells were seeded into 96-well plates and treated with 10µM of each LOPAC compound, this was done in triplicate wells. The wells at the edge of the 96-well plate were left untreated and not used as controls. All the molecules were dissolved in DMSO so triplicate wells were also treated with the same volume DMSO as controls. Cells were incubated under their normal growth conditions for 48 hours after which reporter splicing was determined using a fluorescent plate reader to measure the

levels of dsRED and EGFP expression in individual wells of the 96 well plate. The triplicate measurement recorded for both dsRED and EGFP were compared to control and statistically analysed using a one-way ANOVA. Each treated condition was compared to DMSO control using Dunnett's post-test. As it was aimed to identify molecules that can reduce the pro-angiogenic VEGF proteins or increase the anti-angiogenic isoforms, compounds that caused a dsRED reduction or GFP increase compared to control with a P-value<0.05 were selected for further investigation.

The primary screen yielded 299 small molecules that caused a significant increase in EGFP measurement, 34 that had reduced dsRED and 36 that had a significant effect on both fluorescent proteins. In theory, a switch in alternative splicing of VEGF SSFR transcripts from predominant use of the proximal splice site to the distal splice site should produce an increase in EGFP and reduce dsRED. As the majority of changes in fluorescent protein levels were increases in EGFP, it is possible there is some bias in the screen with small decreases in dsRED more difficult to detect using this method. As such, chemicals that produced EGFP-only increases were also selected to be investigated further as part of control and secondary screening.

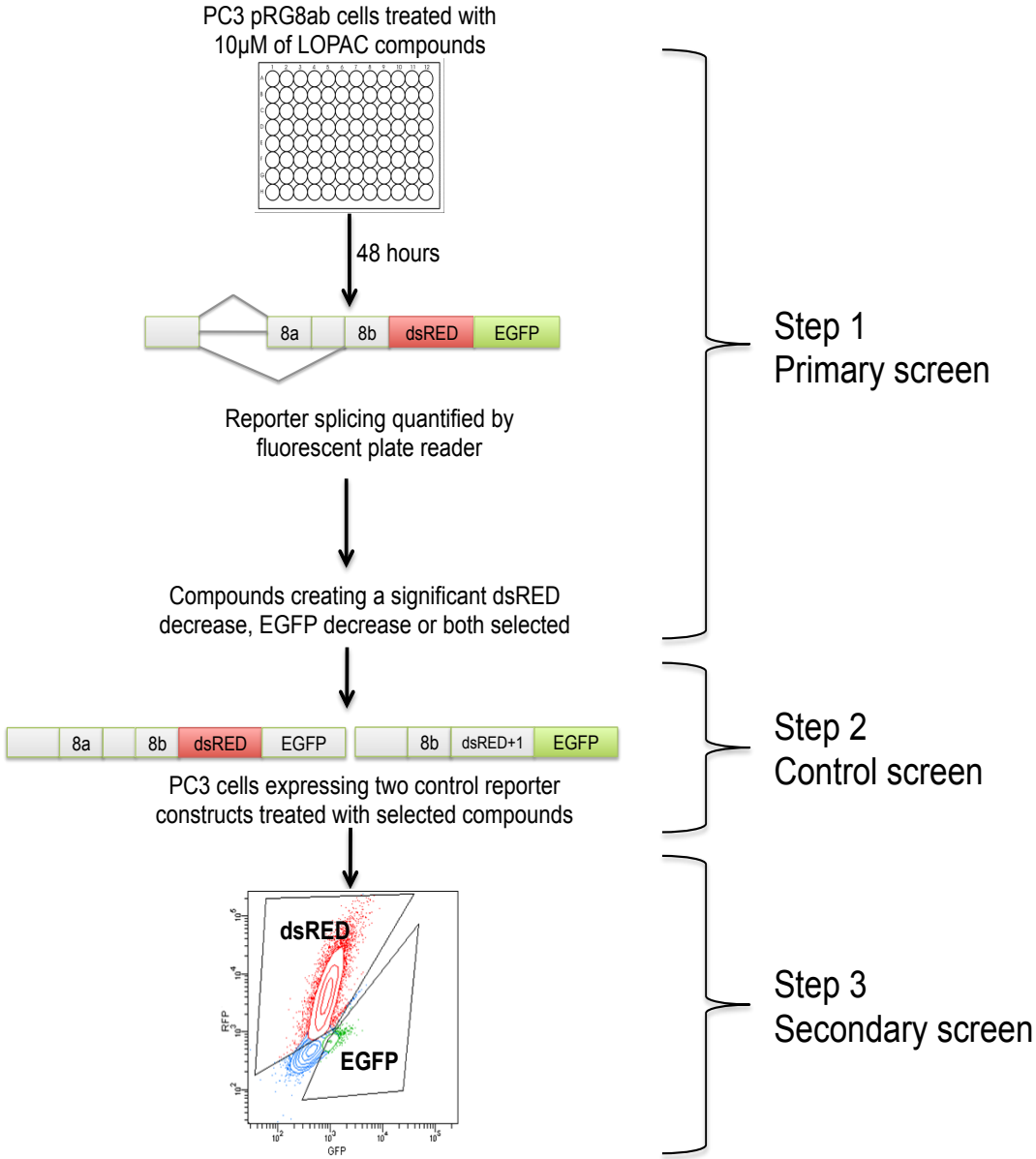
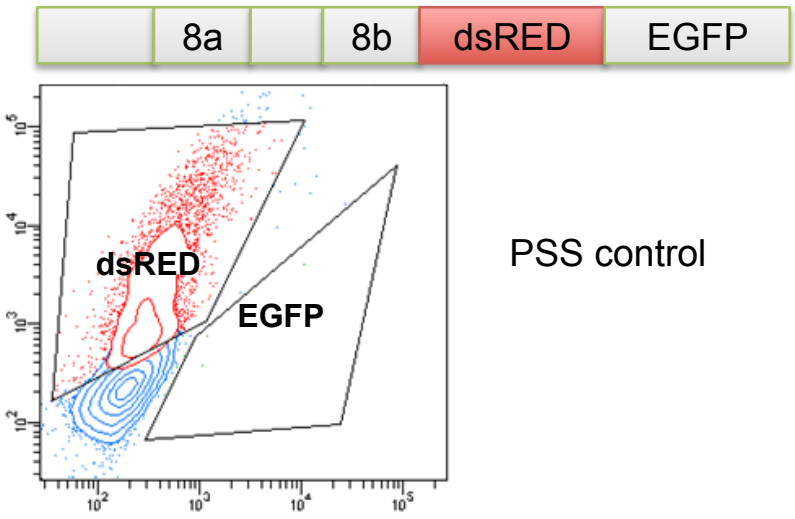


Figure 4-2 Screening the LOPAC library for small molecules that effect the alternative splicing of the VEGF reporter construct.
PC3 cells transfected with the fluorescent VEGF splicing reporter were treated with the LOPAC compound library and reporter AS measured using a fluorescent plate reader (Primary screen, step 1). Hit compounds were used to treat non-splicing control reporter constructs (Control screen, step 2). Flow cytometry was used to measure the effect of the hit compounds on reporter splicing (Secondary screen, step 3).

4.2.2 Using control reporter constructs to eliminate false positive hits

The expression of fluorescent proteins within the reporter transfected cells depend on several factors with alternative splicing being just one. Treatment of the reporter cell line with a small molecule may have unknown effects on fluorescent protein stability or the molecule may be fluorescent itself. This will impact the measurements recorded by the fluorescent plate reader and may have led to false positive hits during the primary screen. To rectify this, two control reporters were designed and cloned (Figure 4-3). The control plasmid DNA did not contain the intron of the original reporter, therefore, mimicked the mRNA transcripts that are produced when either proximal or distal reporter 3' splice site is chosen. The plasmids were termed PSS and DSS control reporters. PC3 cells were transfected with either the PSS or DSS control reporter and selected for plasmid uptake using G418 antibiotic. Flow cytometry was used to confirm expression of each control reporter and fluorescence activated cell sorting was used isolate fluorescent cells (Figure 4-3).

Almost 300 LOPAC compounds induced an increase in EGFP output when used to treat PC3 cells. Due to this high number of molecules, they were ranked according to their significance and the 100 compounds with the smallest p-value were used to treat PC3 PSS and DSS control cells at 10μM for 48 hours. Compounds that significantly reduced dsRED and affected both dsRED and EGFP were also used to treat control reporter cells (Figure 4-2, step 2). The control reporter mRNA cannot be spliced, as there is no intron present, thus any changes in the fluorescence measured by the plate reader were not due to the treatment having an effect on reporter alternative splicing. Again, dsRED and EGFP measurements were statistically compared to control. Compounds that induced a significant difference ($p<0.05$) in fluorescence output were deemed to be false positives and eliminated from further investigation.



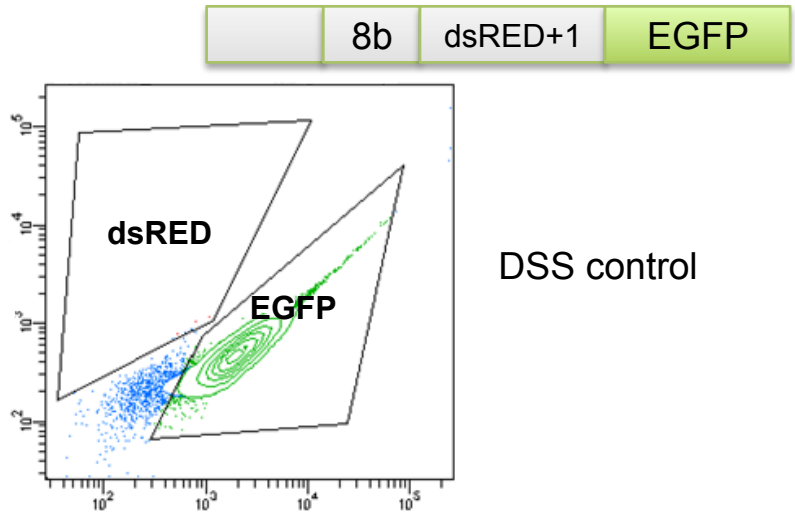


Figure 4-3 Design and expression of proximal splice site and distal splice site control reporters
Two control reporters were designed and cloned to mimic the two mature mRNA transcripts that can be produced from the alternative splicing of pRG8ab pre-mRNA-proximal (PSS) and distal splice site (DSS) controls. Both constructs were transfected into PC3 cells and their expression confirmed by flow cytometry.

4.2.3 A secondary screen for modifiers of reporter alternative splicing

Following the elimination of false-positives, 26 compounds that significantly decreased dsRED signal, 90 that increased EGFP and 19 that caused both were selected. Again, due to the high number of hits causing an EGFP increase, these were ranked according to their difference from the control and the 50 compounds with the greatest difference were used during the primary screen of LOPAC molecules. The selected compounds were tested again in a secondary screen to confirm any effects on reporter alternative splicing (Figure 4-2, step 3). The secondary screen was performed using flow cytometry to measure the expression of fluorescent proteins in cells treated with the hit LOPAC compounds. Cells were treated with each compound at 10μM for 48 hours before collection and flow cytometric analysis (Figure 4-4). Flow cytometry measured the percentage of the cell population that were expressing dsRED or EGFP. This was used to calculate the ratio of dsRED/EGFP. A decrease in dsRED/EGFP indicated reduced use of the reporter proximal splice site and increased distal splice site selection. Out of the 135 compounds tested in the secondary screen (using control reporter cell lines), nine compounds produced a decrease in the ratio of PSS to DSS selection (Figure 4-4). The compounds were given the shortened name ESSO1-9. The names of the chemicals and their effect on dsRED and EGFP during the primary screen (pRG8ab cells) are listed in Table 4.

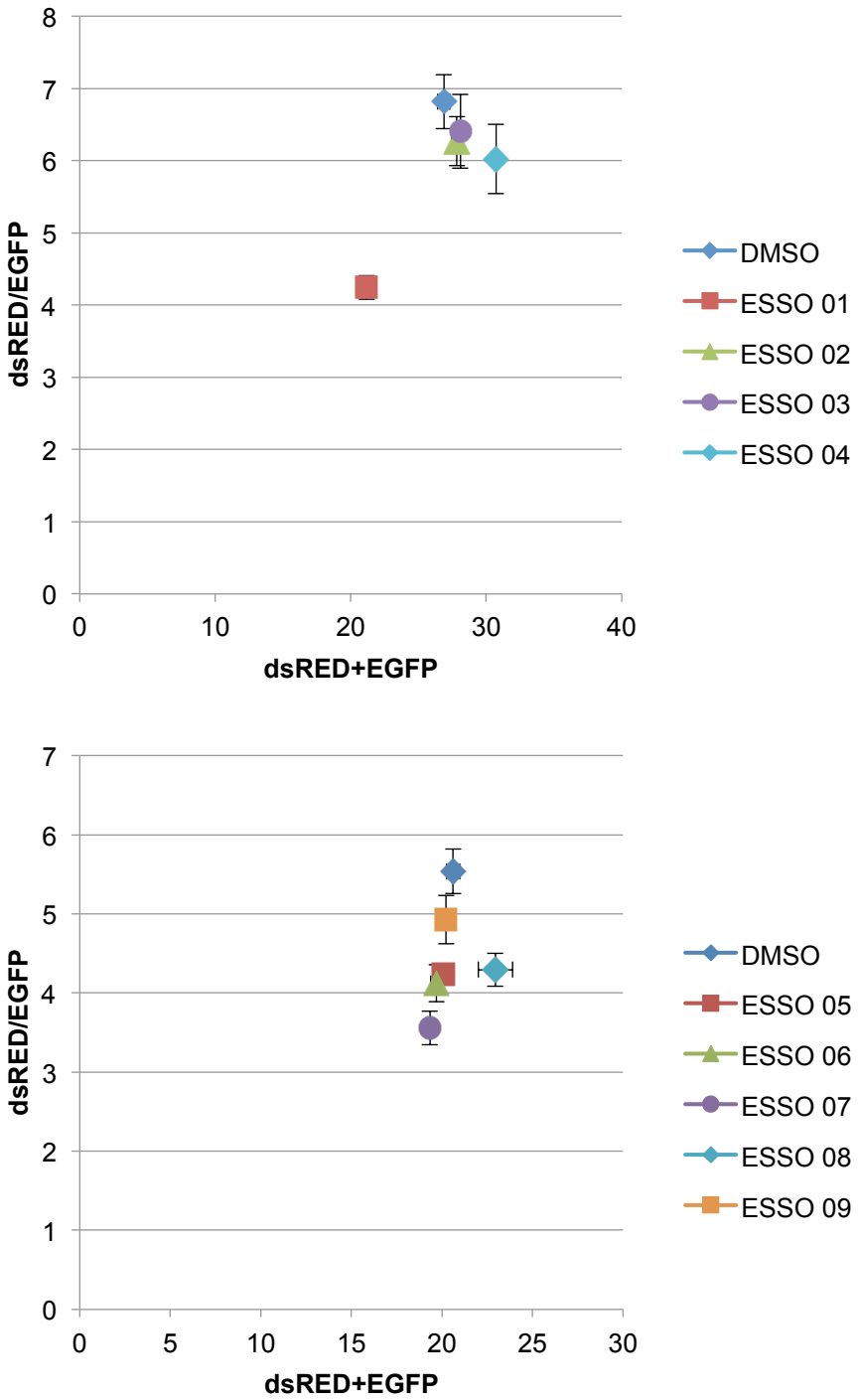


Figure 4-4 A secondary screen of small molecule hits of LOPAC using flow cytometry
PC3 pRG8ab cells were treated with 135 compounds of the LOPAC library for 48 hours. Reporter splicing was measured using flow cytometry. The ratio between the two fluorescent proteins (dsRED/EGFP) measured changes in reporter splice site selection. The sum of the populations expressing dsRED or EGFP was used to show changes in overall expression of the reporter construct.

Compound	Shortened name	EGFP	dsRED
Trovafloxacin mesylate	ESSO01	↑	↓
Melatonin	ESSO02	↑	↓
5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone	ESSO03	↑	↓
N6-2-(4-Aminophenyl) ethyladenosine	ESSO04	↑	↓
8-Bromoadenosine-3',5'-cyclophosphate sodium	ESSO05		↓
Flupirtine maleate	ESSO06	↑	
RepSox	ESSO07	↑	
GW2974	ESSO08	↑	
4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride	ESSO09	↑	

Table 4 Screening compounds shown to reduce the dsRED/EGFP ratio.

4.2.4 The effect of hit compounds on endogenous VEGF alternative splicing

Nine compounds of the LOPAC library were shown to alter the ratio of pRG8ab fluorescent reporter splice site selection. As the reporter is an artificial construct under the transcriptional control of the highly active CMV promoter, it needed to be determined if the small molecules have the same effect on endogenous *VEGF* mRNA alternative splicing and if this then produces increased expression of anti-angiogenic protein isoforms. RNA was isolated from PC3 cells treated with each of the compounds for 48 hours, cDNA was reverse transcribed and amplified by PCR. The primers used for amplification were designed to be able to detect both pro- and anti-angiogenic isoform transcripts with the distally spliced transcripts 66bp small than transcripts that had proximal splice site use (Figure 4-5). The compounds ESSO01 and ESSO07 demonstrated the clearest switch in *VEGF* alternative splicing with increased $VEGF_{xxx}b$ and reduced $VEGF_{xxx}$ transcripts detected.

VEGF anti-angiogenic isoform expression was also gauged at the protein level (Figure 4-6). PC3 cells were treated with four of the lead compounds that were being investigated in greater detail; ESSO01, 07, 08 and 09. Treatment with SPHINX was used as a positive control as the SRPK1 inhibitor is known to affect *VEGF* alternative splicing, inducing exon 8 DSS selection and therefore, increasing the expression of anti-angiogenic VEGF isoforms (Amin et al., 2011; Gammons et al., 2013b; Nowak et al., 2010). Following 48 hours of treatment, protein was extracted from cells and western blotting used to detect anti-angiogenic VEGF isoforms and total VEGF levels. Membranes were probed with an antibody that specifically detects $VEGF_{xxx}b$ isoforms before reprobing with another that binds all VEGF isoforms, both pro- and anti-angiogenic. This provides an indication of changes in *VEGF* alternative splicing. ESSO01 appeared to greatly reduce total protein levels. The other lead compounds reduced total VEGF expression but increased $VEGF_{xxx}b$ isoforms.

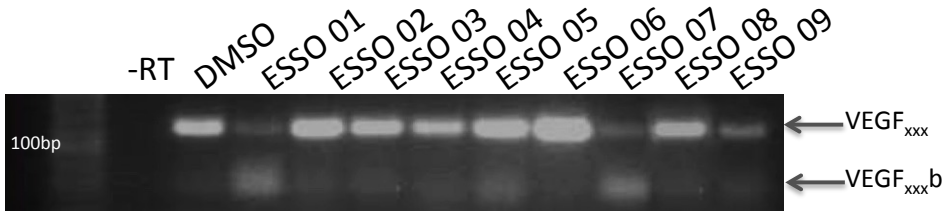


Figure 4-5 The effect of ESSO compounds on the alternative splicing of endogenous VEGF mRNA transcripts
RNA was extracted from PC3 cells, reverse transcribed and amplified by PCR. Anti-angiogenic isoform transcripts were expected to be 66bp smaller than pro-angiogenic transcripts.

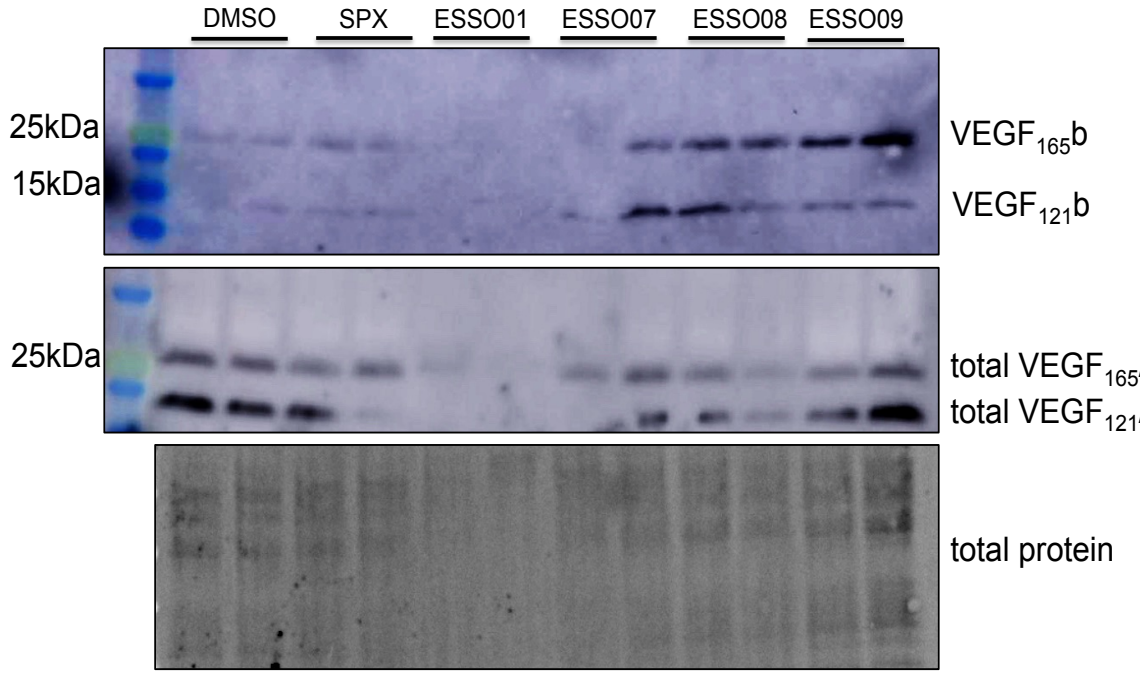


Figure 4-6 Changes in expression of anti-angiogenic VEGF protein isoforms following treatment with ESSO compounds
Following treatment with each small molecule (10uM, 48 hours), protein was extracted and used for Western blotting. The bands observed correspond to VEGF isoforms that are 165 and 121 amino acids in length.

4.3 DISCUSSION

4.3.1 Success and limitations of current anti-angiogenic therapies

When first proposed, anti-angiogenic therapy for cancer treatment was a particularly attractive prospect as all solid tumours require angiogenic vessel sprouting to grow beyond 1-2mm in diameter. VEGF is the key angiogenic driver and overexpressed by most tumour types. Preliminary animal models demonstrated that VEGF inhibition could reduce tumour growth. This resulted in various anti-cancer agents being developed to target VEGF and the VEGF signalling pathways. Several classes of anti-angiogenic drugs have been produced. Agents that directly bind and inhibit VEGF include bevacizumab and aflibercept. Other drugs target the VEGF receptors, such as the antibody ramucirumab that binds VEGFR2. Small molecule receptor tyrosine kinase inhibitors (TKIs) have also been used to block the signalling activity of VEGFRs. The TKIs developed to inhibit VEGFRs do also have off target effects on PDGFRs and FGFRs. Anti-VEGF therapy has so far had relatively limited success in combating cancer. Some of the treatments have shown efficacy in certain cancers. As angiogenesis does not commonly occur in the healthy adult, it was presumed that drugs targeting VEGF would avoid major issues of toxicity. However, reported adverse effects during anti-angiogenic therapy include hypertension, proteinuria, haemorrhaging, endocrine defects and gastrointestinal perforations (Vasudev and Reynolds, 2014).

Despite angiogenesis being essential for the continued growth of solid tumours, only a small number of cancers have been approved for treatment with anti-angiogenic therapeutics. Some patients develop resistance to anti-VEGF agents. Certain tumours respond well, with reduced perfusion of tumour blood vessels and reduction of tumour volume. In others, the number of vessels is reduced but the tumour is stabilised and does not regress. Many tumours have intrinsic resistance to anti-angiogenics and continue to grow during treatment, with insensitive vasculature. Some tumours demonstrate acquired resistance whereby the tumour initially respond but is followed by further growth either with or

without angiogenic sprouting. Continued angiogenesis during treatment with anti-VEGF drugs may be due to adaption and activation of alternative pro-angiogenic pathways. The vasculature of a tumour is heterogeneous and vessels vary in their response to VEGF inhibitors. Pre-clinical data suggests newly formed vessels within a tumour are sensitive to anti-VEGF agents but as vessels mature some lose dependency on VEGF and are therefore less responsive. Tumour vessels are abnormal, tortuous and grow aberrantly. It has been suggested that therapies should attempt to normalise tumour vessels to improve their perfusion and optimise exposure of the tumour to chemotherapeutics (Jain, 2005).

Many cancer cells secrete high levels of pro-angiogenic VEGF, stimulating the growth of new vessels to nourish the tumour. The $VEGF_{xxx}b$ family of sister isoforms are anti-angiogenic, have reduced expression in tumour tissues and can reduce tumour growth. $VEGF_{165}b$ possesses binding domains that will associate with most anti-VEGF targeted antibodies, including therapeutic bevacizumab. $VEGF_{165}$ and $VEGF_{165}b$ bind bevacizumab with the same affinity, but the ability of bevacizumab to reduce tumour growth is affected by $VEGF_{165}b$. In mice, bevacizumab did not have the same inhibitory effect on tumour growth when cancer cells were expressing $VEGF_{165}$ b. This has implications for anti-VEGF therapy (Varey et al., 2008a). New ways of targeting tumour angiogenesis must be found and exploited. One potential method would be to not directly target VEGF protein or VEGF expression but to control its alternative splicing to produce $VEGF_{165}b$ as an endogenous anti-angiogenic agent.

4.3.2 Screening for novel regulators of VEGF alternative splicing

This screen searched for small molecules that could alter *VEGF* alternative splicing- reducing the splicing pattern that creates pro-angiogenic VEGF isoforms and increasing the anti-angiogenic isoforms. The library used for this screen, LOPAC, was a collection of FDA-approved compounds with known pharmacological activities. This is an example of drug repositioning. Drug repositioning allows established drugs to be investigated and find new uses in alternative diseases. It can also deepen our insight into the mechanisms that

cause pathogenesis and could facilitate more rapid transfer of drugs into clinical trials. Repositioned drugs will have already endured toxicity and patient safety assessment whereas this will still need to be completed for potential therapeutics that are identified using traditional high-throughput screening libraries (Wilkinson and Pritchard, 2015).

There have been a handful of instances in which drugs previously used for a different disease have been found to be useful in treating certain cancers. A well-known example is thalidomide. Patients with leprosy that developed painful lesions on their skin were given thalidomide for its sedative properties. The quantity of lesions was decreased by thalidomide. Thalidomide has since been used as a therapeutic for multiple myeloma and been shown to be anti-angiogenic (D'Amato et al., 1994; Singhal et al., 1999).

There are also various examples of drug repositioning candidates being identified using *in vitro* screens in a range of diseases including cancers. It has been shown that cell death can be stimulated in small cell lung cancer cells by tricyclic antidepressants (Jahchan et al., 2013). Another example is the drug mebenozole, which was identified as causing toxicity in cell lines from colon cancer. Mebenozole is normally used as a treatment for infection with gut worms but is now known to also inhibit kinases including B-Raf (Nygren et al., 2013). Prostate cancer cells, PC3 and LnCap, were treated with a drug library and analysed for proliferation and DNA synthesis. Digoxin, an anti-arrhythmic agent, was found to inhibit proliferation in these cells. An epidemiological analysis found the risk of developing prostate cancer was lower in men who had used digoxin (Platz et al., 2011)

4.3.3 Statistical analysis of screen data

In large-scale high throughput screens, there are several statistical methods commonly employed to identify hits from the noise. Routinely during a primary screen that tests millions of compounds, the activity of each compound will only be measured once, without replicates. This limits the statistical testing that can

be applied to the data. The 'Z-score' method is often used to select positive hits from large data sets. Z-scoring does not use control measurements but assumes that most compounds within a screening library will not be active within the assay so can be used as controls. In this case, the average measurement from within a plate is subtracted from the compound measurement and divided by the standard deviation from the entire plate. Alternatively, the 'percentage of control' method normalises compound activity relative to controls by dividing raw compound measurements by the average of controls within each plate. Measurements can then be compared across many different plates. Both these methods assume randomly distributed error but this cannot be confirmed without replicate measurements of compound activity. Statistical outliers will also affect the mean and standard deviation across a plate.

As the compound library used in the screen for pRG8ab splicing modulators was relatively small, it was feasible to include replicates within the screen allowing the calculation of error and analysis of variance. The use of replicates was decided on at cell-based assays can encounter clumping and uneven distribution of cells between wells. Evaporation of growth media may also occur across areas of the 96-well plate altering growth conditions and leading to bias depending on the well position. Some false positives within screens are known to be caused by positional bias. To reduce this, each compound was used to treat three wells of a 96-well plate. A one-way AVOVA was performed on the dsRED and EGFP measurements using DMSO reading from the same plate as the internal control measurement. Fluorescence readings were normalized to the DMSO control measurement from the same plate. As the screen was performed manually, with readings taken over many different days, it was more appropriate to use the DMSO of each plate as the control measurement rather than an average of the DMSO readings over all plates used.

4.3.4 Limitations and false positive elimination within fluorescent reporter assays

As with all drug discovery techniques, screening has limitations and down sides. Cell-based assays are a useful tool in the process of HTS but the potential for variation and noise is increased by the use of live cells. This includes variation in cell growth and response between different plates and uneven measurements within the same plates. One such technical issue is the 'edge effect'. It is well established that luminescence or fluorescent measurements detected from wells at the edge of 96-well plates are often considerably lower or higher than the rest of the plate. This is possibly cause by a thermal gradient between the outer-most wells and those in the middle of the plate (Lundholt et al., 2003; Malo et al., 2006). This may result in uneven distribution cells or altered adhesion and morphological changes. During the LOPAC screen, cells were seeded into the outer wells put were not treated or used as control measurements to avoid the edge effect.

The primary screen produced 369 'hit' compounds that caused a significant increase in EGFP, decrease in dsRED or both. Out of these 369 chemicals the majority, 299, had induced a significant increase in EGFP measurement. This suggests there may be bias within the plate reader screen, with changes in EGFP more readily detectable. During the secondary screen, fluorescent protein expression was detected by flow cytometry. Nine compounds induced a decrease in the fluorescent protein ratio, dsRED/EGFP. Out of these nine compounds, four had been selected from the plate reader screen because of an increase in EGFP and dsRED decrease. One small molecule had induced a reduced dsRED measurement only and the other four had increased EGFP with no effect on dsRED. Overall, eight out of nine of the compounds that reduced dsRED/EGFP during the secondary screen had increased EGFP during the primary screen. Again, this indicates there is some inherent bias either from the fluorescent plate reader or the reporter construct itself whereby changes in dsRED are more difficult to detect, harder to induce or there are differences in the half-lives of the fluorescent proteins.

Small molecule screens have been used previously to elucidate compounds that can change the inclusion of an exon or the use of an alternative 5' or 3' splice site. Several screens have used splicing reporters with luciferase or GFP output (Warzecha et al., 2009). Reporters such as these can be useful but do contain limitations, for example, the effect of a small molecule on reporter alternative splicing cannot always be differentiated from effects via transcriptional and translational mechanisms. Dual colour fluorescent splicing reporters should be less likely to suffer from false positives caused by transcription/translational modifiers as true splicing switches should cause an increase in one fluorescent protein and a decrease in the other.

Stoilov et al., 2008 used a dual colour splicing reporter system to screen for small molecules that can modify the alternative splicing of MAPT. They found that some compounds that altered overall gene expression caused changes in the ratio between the two fluorescent proteins of their reporter. They concluded that this was caused by the fact that half-lives of their RFP and GFP were different. Transcription and splicing inhibitors also were a source of false positives likely caused by the differing stabilities of the two mRNA transcripts produced from alternative splicing of the reporter. Additionally, if the compound is a strong fluorophore, which fluoresces in the same range as the measured fluorescent protein, it will also produce a false positive.

In the VEGF SSFR screen, the issues regarding false positives were overcome through the use of the two control reporters (Figure 4-3). The control reporters could not be spliced as they lacked an intron but exactly mimicked the mature mRNA transcripts produced after alternative splicing of RG8ab pre-mRNA. Any compounds that alter fluorescent protein expression during the primary screen via transcriptional or translational mechanisms or by changing transcript/protein stability will have the same effect on the control reporter constructs. Whereas small molecules that caused a true splicing switch will not have had any effect on the control reporters- there is no intron therefore, no splicing.

There are also other important forms of gene expression regulation beyond transcriptional control and alternative splicing, which could affect reporter transcripts and ultimately fluorescent protein measurements. One such mechanism is unproductive splicing, a process used to down regulate the expression of certain alternatively spliced transcripts post-transcriptionally by inducing nonsense-mediated decay (NMD). NMD involves recognition of mRNAs that contain premature termination codons (PTCs) and induces degradation of the transcript for translation into what would be a truncated protein product. Some details of how NMD machinery detects PTCs and targets the mRNA for degradation remain unclear. Nonsense mutations and frameshifts can produce mRNAs containing PTCs, as can use of alternative splice sites (Lareau et al., 2004). Computational analysis of alternatively spliced mRNAs indicates that of human genes known to undergo alternative splicing, around 45% produce one or more mRNAs containing a PTC making it a target for NMD. This gave rise to the notion that unproductive splicing is not simply an irregularity, but a regulated mechanism of post-transcriptional gene regulation (Lewis et al., 2003). Aside from inducing mechanisms that favour selection of either the VEGF SSFR proximal or distal splice site, some compounds from the chemical library may induce use of another sequence within the reporter construct as a splice site. These transcripts could contain PTCs resulting in degraded by NMD. This could act to decrease dsRED expression from the reporter without a splicing switch to the distal splice site. The control reporters used as part of the VEGF SSFR screen cannot be spliced at the *VEGF* proximal and distal splice sites as the *VEGF* exon 7 intronic sequence has been removed. Processing of the control reporter transcripts could still potentially produce products that are targeted for degradation and these would not be eliminated during the control screen.

Chapter 5

Anti-angiogenic effect of compounds identified using VEGF splicing sensitive reporter

5.1 INTRODUCTION

Angiogenesis is the physiological process of blood vessel growth from pre-existing vasculature. Angiogenesis is essential during embryogenesis and development for the growth of organs and tissues but does not commonly occur in adult life, apart from a few notable exceptions such as during wound healing processes. The female reproductive system also undergoes periods of physiological angiogenesis during follicle development in the ovary and within the placenta during pregnancy (Carmeliet, 2005).

Endothelial cells form the lining of all blood vessels. Under basal conditions, endothelial cells are quiescent and their survival is maintained by many autocrine factors including angiopoietins, FGFs and VEGFs. These signals provide the quiescent endothelial cells with long half-lives. Endothelial cells of the microvasculature are arranged in monolayers covered by pericytes, which also secrete survival signals and repress the proliferation of endothelial cells. When a vessel is exposed to an angiogenic signal, pericytes become detached from the basement membrane and the junctions between endothelial cells are weakened. This makes the vessel more permeable and proteins move from the plasma to create additional extracellular matrix for endothelial cells to form new vascular structures on. ECM proteins are cleaved by proteases to release angiogenic factors and to allow remodelling (Carmeliet and Jain, 2011).

During angiogenesis, one endothelial cell from the stimulated vessel is chosen as the tip cell, which leads sprouting vessels towards an angiogenic stimulus. The adjacent cells become stalk cells, which proliferate to elongate the new vessel.

After angiogenesis, endothelial cells return to quiescence with the cell-to-cell junctions reforming and pericytes stimulated to sheath the new vascular tubules. For such a complex process, it is surprising that one family of proteins, VEGF-A, regulates angiogenesis so prevalently (Carmeliet, 2005).

Angiogenesis is highly regulated at each step. To investigate angiogenesis, assays need to simulate each important stage of the process to be used to effectively evaluate potential therapeutics. *In vitro* assays designed to explore vessel growth often focus on one phase of angiogenesis i.e. endothelial cell proliferation, migration etc. Endothelial proliferation can be assessed by measuring the incorporation of radiolabelled nucleotides or expression of proliferative markers, such as Ki-67. Boyden chamber assays can be used to measure endothelial cell migration in response to a stimulus. In such assays, cells are cultured on a porous filter with their movement through the filter used as a quantification of their migratory phenotype (Auerbach et al., 2003; Ferrara, 2002).

Another common way to evaluate the effect of a treatment on endothelial cells and angiogenesis *in vitro* is using tube formation assays. This involves culturing ECs on a matrix of extracellular proteins. All cultured endothelial cell lines are able to adhere to the gelatinous matrices and form tube-like networks mimicking capillaries of the microvasculature. A commonly used matrix is Matrigel, produced from mouse sarcoma Engelbreth-Holm-Swarm cells. Similarly, tube formation assays of endothelial cells can also be performed in co-culture with other cells such as fibroblasts. During the co-culture, fibroblasts secrete ECM proteins, which endothelial cells then use to form tubes *in vitro*.

In vitro angiogenesis assays are an important step in validating drugs or determining which genes can alter angiogenesis. But, due to the complexity of vessel growth, there is no way to fully simulate angiogenesis *in vitro*, therefore, *in vivo* models must also be employed. An example of one such assay is the corneal pocket angiogenesis assay. The cornea is a transparent tissue and normally has no vasculature making it easy to observe neovascularation caused

by an injury or injection of tumour cells into the corneal pocket. Another common method used to investigate angiogenesis *in vivo* involves subcutaneously injecting an animal with a Matrigel plug. The Matrigel can be mixed with a potential pro/anti-angiogenic molecule or cells expressing angiogenic factors, which may stimulate or inhibit vessel growth and infiltration into the plug. After several days the plug can be removed and vessel growth within the plug quantified using endothelial markers (Staton et al., 2009).

In the previous chapter I described a chemical screen, which used a reporter construct to identify molecules that may influence *VEGF* alternative splicing. Some of the chemicals were shown to increase expression of anti-angiogenic VEGF protein isoforms. Here, I investigate whether these compounds produce an anti-angiogenic effect and if this effect is mediated by VEGF isoforms.

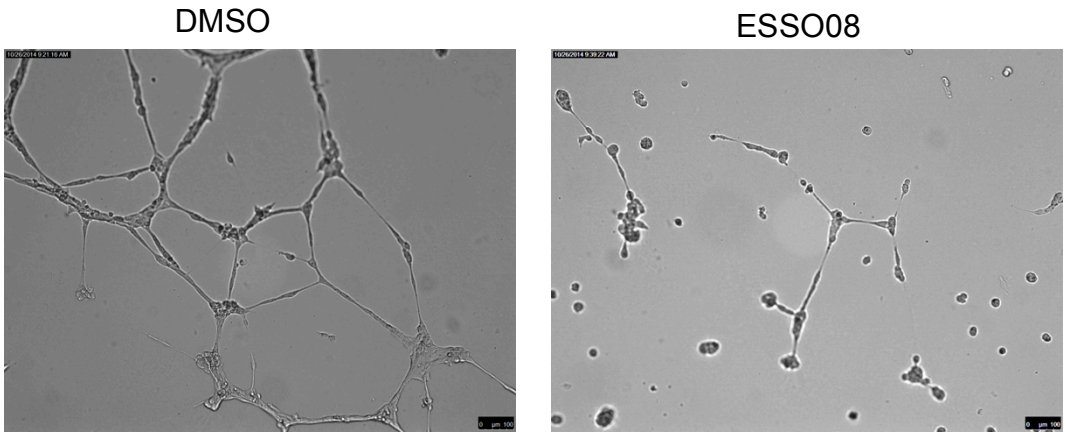
5.2 RESULTS

5.2.1 The effect of ESSO compounds on endothelial cell tube formation on Matrigel

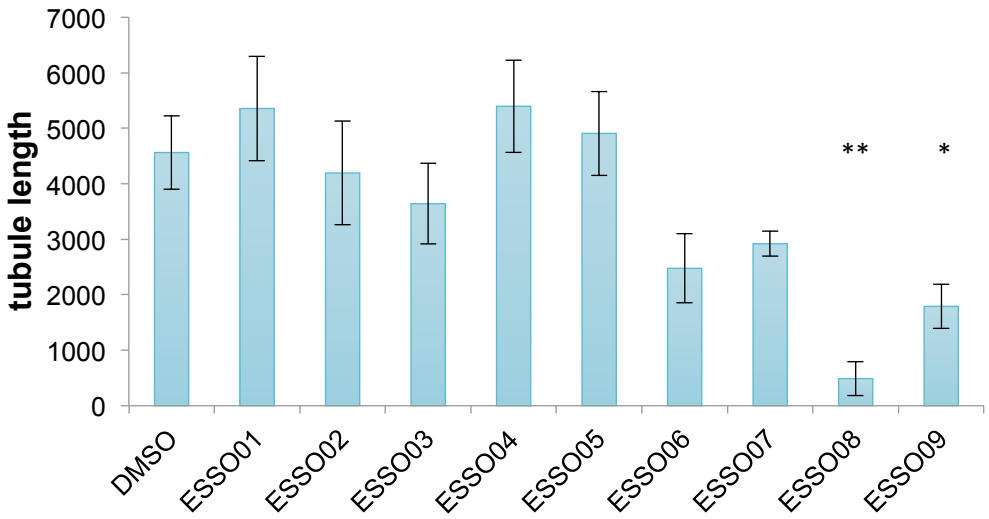
A library of compounds was used in a screen to identify molecules that can alter *VEGF* alternative splicing using a splicing-sensitive fluorescent reporter that mimics *VEGF* terminal exon splicing. This splicing decision creates either pro- or anti-angiogenic VEGF protein isoforms. Nine compounds were shown to increase distal splice site selection/ reduce proximal splice site selection following a primary and secondary screen of a 1280 compound library. Hits had been shown to change the alternative splicing of a reporter based on *VEGF* exon 8, increasing use of the distal splice site involved in producing anti-angiogenic VEGF. Some of the lead compounds affected the alternative splicing of endogenous *VEGF* mRNA transcripts in PC3 cells and increased expression of anti-angiogenic VEGF isoforms at the protein level. Continuing, I wanted to establish if any of the compounds produced an anti-angiogenic response. I chose to investigate this by performing several anti-angiogenesis assays *in vitro* and *in vivo* including Matrigel tube formation assays, endothelial-fibroblast co-culture and *in vivo* Matrigel assays.

Human umbilical vein endothelial cells (HUVECs) were cultured in for 48 hours in EBM-2 media containing either control (DMSO) or one of the nine drugs. Cells were then detached from culture plates and 10,000 HUVECs seeded into each well of a 96-well plate coated with Matrigel and incubated at 37°C. Micrographs were taken of cells in each treatment condition after five hours of incubation (Figure 5-1A). Five images were captured of each well. The total length of tubules formed in each field was measured using ImageJ (Figure 5-1B) and the number of branch points between tubules counted (Figure 5-1C). ESSO08 (GW2974) and 09 (AEBSF) significantly reduced the tube length and branch points compared to control.

A


DMSO
ESSO08

B


tubule length
7000
6000
5000
4000
3000
2000
1000
0
**
*
DMSO
ESSO01
ESSO02
ESSO03
ESSO04
ESSO05
ESSO06
ESSO07
ESSO08
ESSO09

C

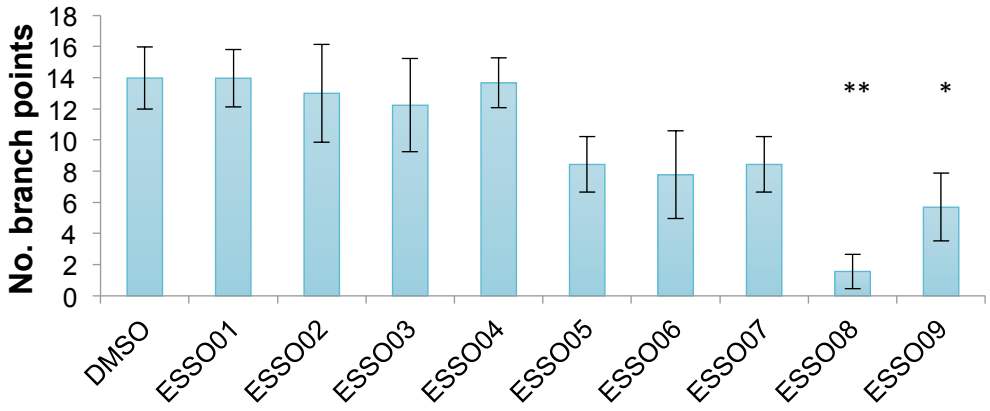


Figure 5-1 The effect of screened lead compounds on endothelial cells in an in vitro angiogenesis assay
*HUVECs were treated with each of the lead compounds for 48 hours at 10µM before being seeded onto Matrigel basement membrane matrix. Cells were imaged after 5 hours (A), 5 microscopic fields were captured of each condition at 10x magnification. Total tubule length (B) and the number of branch points (C) in each image. Statistical test performed one-way ANOVA, with Dunnett's post-test comparing each treatment to DMSO control.*p<0.05, **p<0.01 n=3.*

HUVECs were used in the described *in vitro* angiogenesis assay. During the Matrigel tube formation assay, two compounds were observed to significantly reduce the ability of endothelial cells to form tubular networks. Trypan blue staining was used confirm that the reduction in endothelial tube formation was not merely due to treatment with the four compounds having a detrimental effect on cell viability. HUVECs were treated with each lead compound at 10µM for 48 hours. Cells were then detached from the culture flasks and stained with trypan blue and loaded into a haemocytometer. Viable cells exclude the trypan blue chromophore, therefore remain unstained. Cells were counted to measure cell viability following each compound treatment (Figure 5-2). There was no significant difference in the percentage of viable cells between DMSO control and treatment with any of the nine lead compounds.

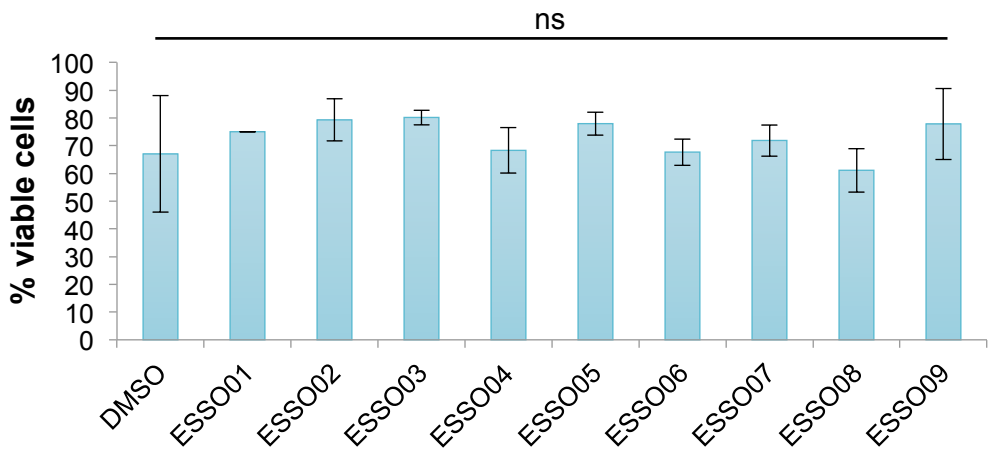


Figure 5-2 Endothelial cell viability is not altered by treatment with lead compounds
HUVECs were cultured in growth media containing either DMSO or one of the nine lead compounds at 10µM. Cells were suspended in a trypan blue solution. Total cell number and viable cells were counted to calculate the percentage of viable cells after treatment. Statistical test performed one-way ANOVA, with Dunnett's post-test comparing each treatment to DMSO control. ns= not significant. n=3.

In the previous tubule formation experiment, endothelial cells were directly treated with the small molecules over 48 hours before the functional assays were performed. During cancer progression, the angiogenic switch, is induced by the secretion of factors from tumour cells that stimulate endothelial cells. Pro-angiogenic VEGF isoforms are highly expressed by tumours and cancer cell lines. Therefore, the small molecule screen, which identified compounds that altered VEGF reporter alternative splicing, was performed using PC3 cells, which normally express pro-angiogenic isoforms. In order to mimic the conditions of the small molecule screen and mimic the *in vivo* situation, PC3 cells were cultured in EBM-2 growth medium and treated with each of the small molecules at 10µM. The conditioned media was removed from PC3 cells after 48 hours of treatment and used in a Matrigel angiogenesis assay. HUVECs were suspended in the conditioned media and seeded onto Matrigel basement membrane matrix. Tubule length in each condition was quantified as a measurement of the angiogenic effect after 5 hours of culture in the conditioned media (Figure 5-3).

Conditioned media of 8 compounds significantly reduced tubule formation by endothelial cells.

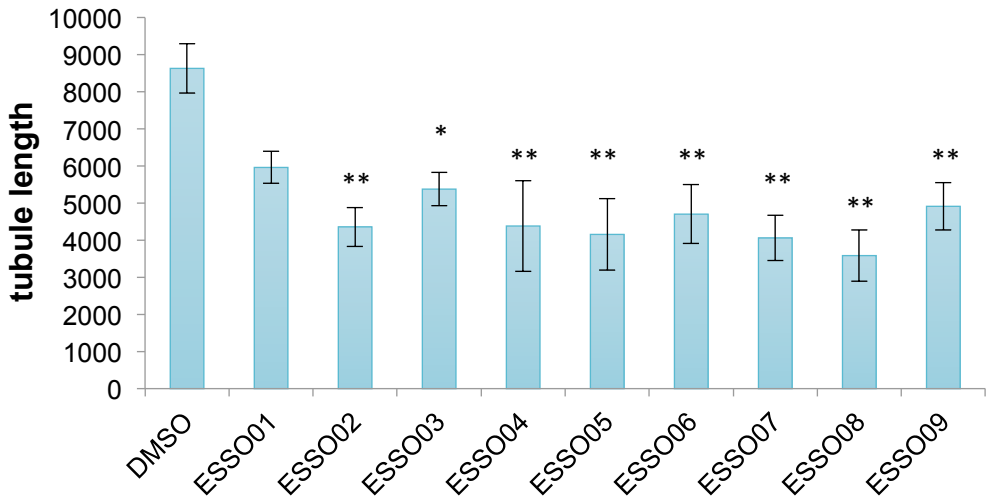


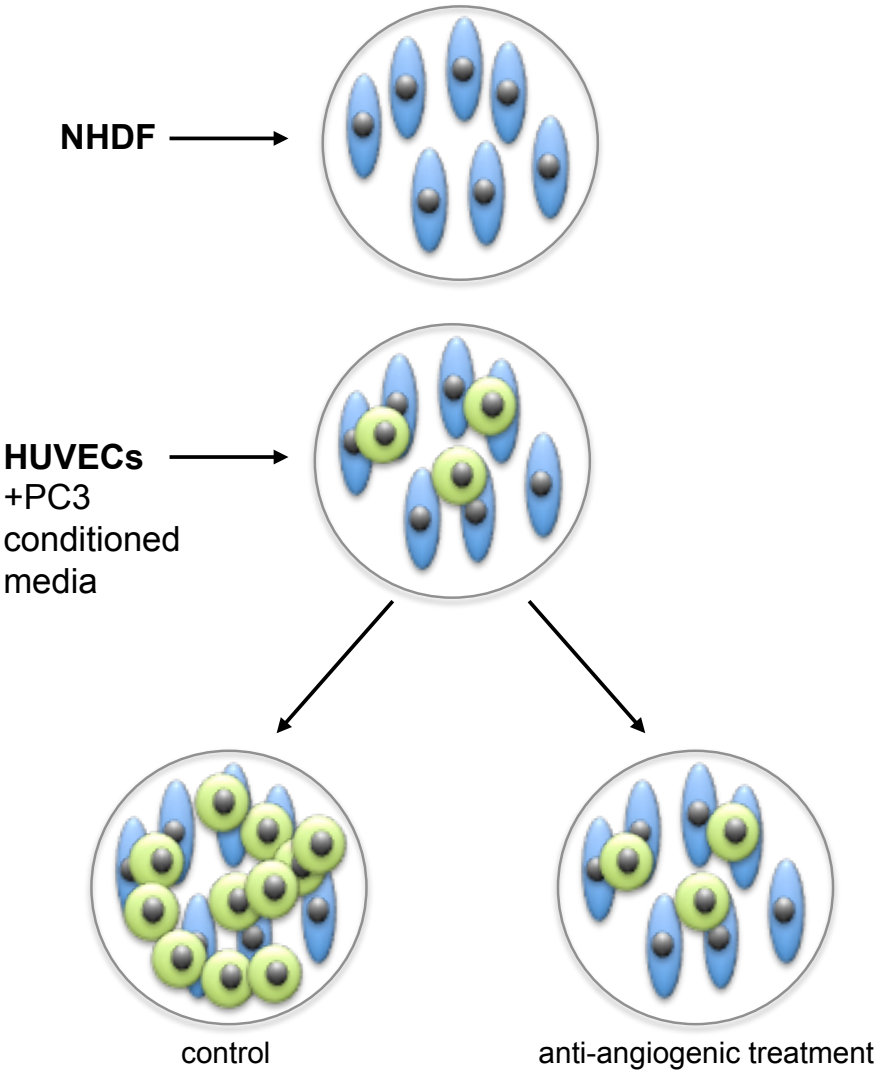
Figure 5-3 Endothelial cell function is altered by the conditioned media of PC3 cells exposed to lead compounds
*PC3 cells were cultured in EBM-2 containing 10μM ESSOs with media collected after 48 hours. 1x104 HUVECs were suspended in each conditioned medium and seeded onto Matrigel. Cells were imaged after 5 hours with 5 microscopic fields w captured of each condition. Total tubule length (B) in each image was quantified. Statistically tested using a one-way ANOVA, with Dunnett's post-test comparing each treatment to DMSO control.*p<0.05, **p<0.01, n=5.*

5.2.2 Endothelial tube formation was reduced by trovafloxacin mesylate and RepSox in an endothelial-fibroblast co-culture

The potential anti-angiogenic effects of the lead compounds identified during the VEGF splicing screen was investigated further using a second *in vitro* angiogenesis assay. The effect of lead compounds on prostate cancer cells and how secreted factors alter angiogenesis was investigated using a co-culture of endothelial cells and fibroblasts. In this assay, endothelial cells use the extracellular matrix proteins secreted by fibroblasts as a scaffold on which to form vessel-like tubules (Figure 5-A).

As previously described, PC3 cells were cultured in EBM-2 growth medium and treated with each of the lead compounds at 10µM. The media was collected after 48 hours of treatment. Fibroblasts were cultured on coverslips with HUVECs seeded when fibroblasts reached confluency. Conditioned media was applied to the co-culture 48 hours after the addition of the HUVECs and cells were incubated for a further 48 hours (Figure 5-4A). Triplicate wells were used for DMSO control and each treated condition. Treatment with 1nM $VEGF_{165}$ recombinant protein was used as a positive control for endothelial tube formation (data not shown). The coverslips were fixed and stained with a fluorescent antibody against CD31, an endothelial cell marker (Figure 5-4B). Five fluorescent micrographs were taken of each control and treated conditions. The total length of endothelial cell tubes formed in each image was quantified and normalised to the total number of cells using nuclear staining with DAPI. Two drugs out of the nine lead compounds (trovafloxacin mesylate and RepSox) produced a decrease in the length of tubes formed compared to DMSO control (Figure 5-4C). Under these two treatments, endothelial cells mostly grew in small islands of cells without branching or any tubular structures created on the extracellular protein scaffolds.

A


NHDF
HUVECs
+PC3
conditioned
media
control
anti-angiogenic treatment

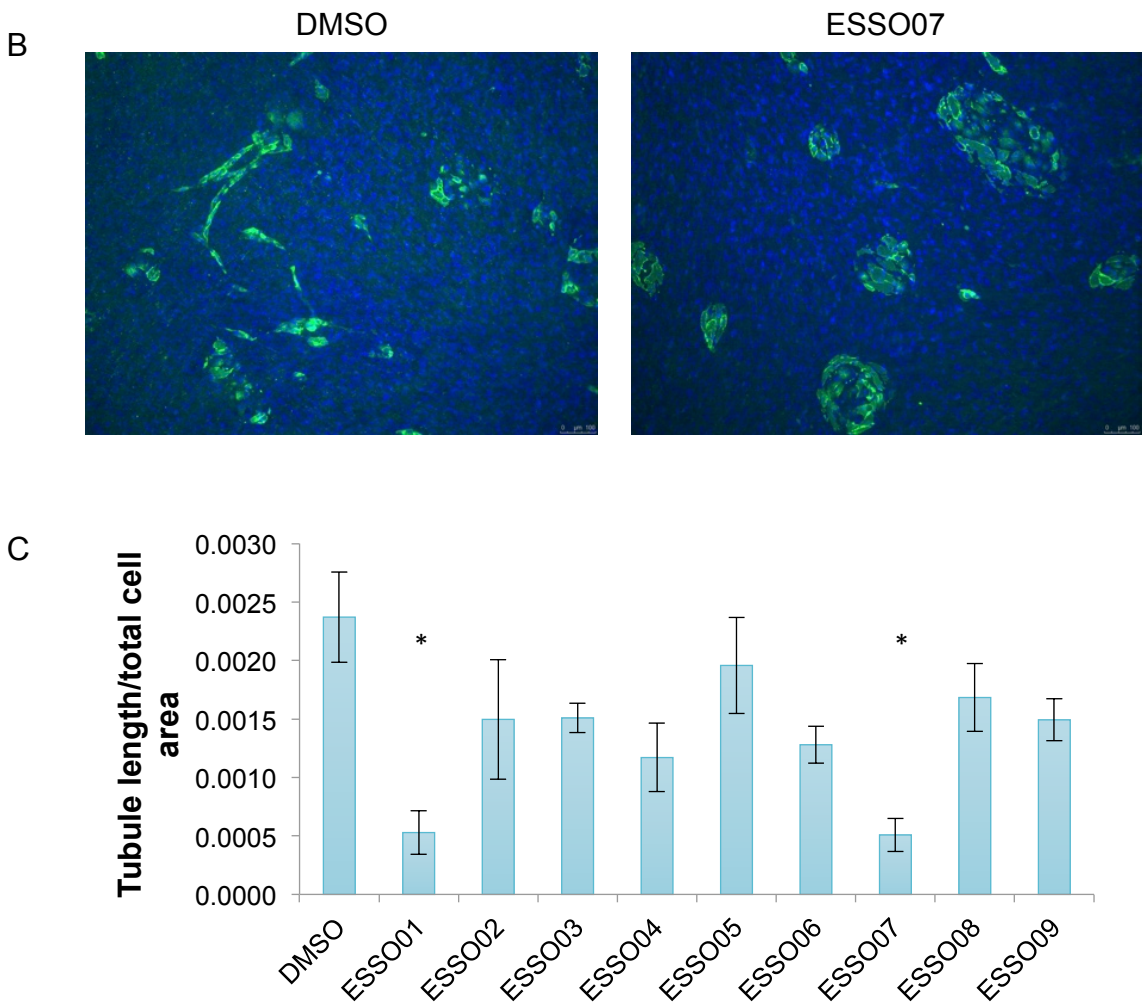


Figure 5-4 Endothelial cell tubule formation was reduced by trovafloxacin mesylate and RepSox during endothelial fibroblast co-culture.
*(A) Endothelial cells were co-cultured with confluent fibroblasts (NHDF) in conditioned media taken from PC3 cells treated with each of the lead compounds (10µM). (B) Cells were fixed and stained with DAPI and a fluorescent antibody against the endothelial cell marker CD31 (C). 10 microscopic fields of each co-culture were quantified for the total length of endothelial tubules formed normalised to the total cell area. Statistical test performed one-way ANOVA, with Dunnett's post-test comparing each treatment to DMSO control. **p<0.01, n=3.*

5.2.3 *Is the ESSO* in vitro *anti-angiogenic activity sole mediated by changes to VEGF alternative splicing?*

I have shown that several of the compounds affect *VEGF* alternative splicing in cultured PC3 cells leading to increased expression of anti-angiogenic VEGF isoforms (Chapter 4). Culture media removed from PC3 cells exposed to the lead compounds reduced tubule formation by endothelial cells following treatment with 8 out of the 9 compounds being investigated. The chemicals all target different molecules so will likely activate different pathways in the cells. The inhibitory effect on the tube forming ability of endothelial cells may be caused by increased $VEGF_{xxx}b$, changes in expression of other secreted factors or from the compounds directly. To assess if $VEGF_{xxx}b$ is the main effector, the Matrigel angiogenesis assays were repeated using a neutralising antibody (56/1) that specifically targets anti-angiogenic VEGF isoforms. The same protocol as previously described was followed but with either 10µg/ml 56/1 or water control added to each conditioned media sample. Consistently with previous experiments, conditioned media from PC3 cells treated with ESSO07 and 08 (RepSox and GW2974) significantly reduced tubule formation by HUVECs, however the effect of ESSO08 was partially rescued by the $VEGF_{xxx}b$ neutralising antibody (Figure 5-5). This indicates that anti-angiogenic VEGF isoforms contributed to the reduced formation of endothelial tubes following treatment with PC3 conditioned media.

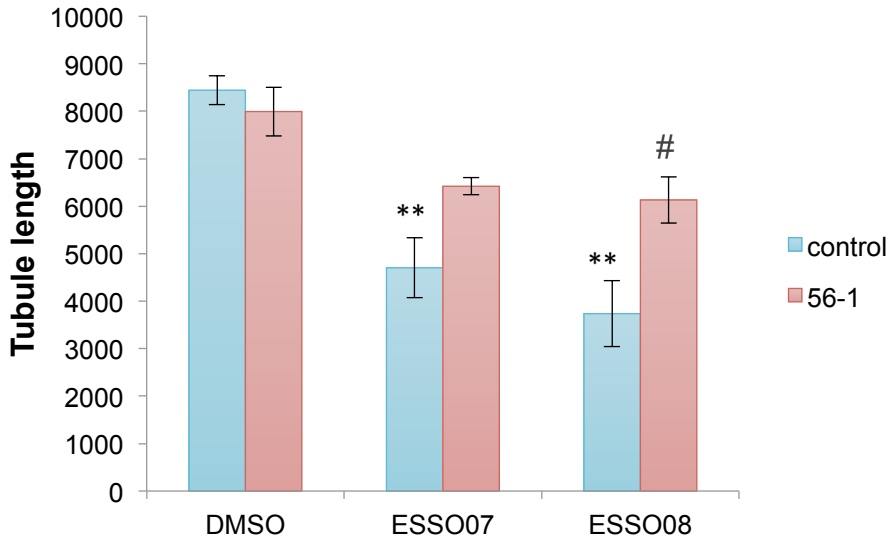


Figure 5-5 Anti-angiogenic VEGF isoforms mediate in vitro inhibition of endothelial tube formation
*PC3 cells were cultured for 48 hours in 10µM of each compound. An endothelial cell tube formation assay was performed with HUVECs seeded on to Matrigel in PC3 conditioned media either containing 56-1 10µg/ml. 5 images of each well were captured and quantified for tubule length. n=4, **P<0.01 vs. DMSO control. # P<0.01 vs. ESSO08 control.*

SR proteins are an important family of splicing regulatory proteins. The phosphorylation and expression of SR proteins following treatment with trovafloxacin mesylate, RepSox and GW2974 (ESSO 1, 7 and 8) was assessed. Cells were pre-treated with the compounds and then stimulated with EGF as EGF has been demonstrated to increase the phosphorylation of SR proteins (Zhou et al., 2012). Proteins were extracted and analysed by western blot. Total SR protein levels were measured and phosphor-SR proteins detected using the mab104 antibody (Figure 5-6). RepSox and GW2974 appeared to reduce the detection of certain phospho-SR bands. Phosphorylation of an approximately 30kDa protein was reduced. This band may represent SRSF1 or 2, which have predicted molecular weights of around 28kDa.

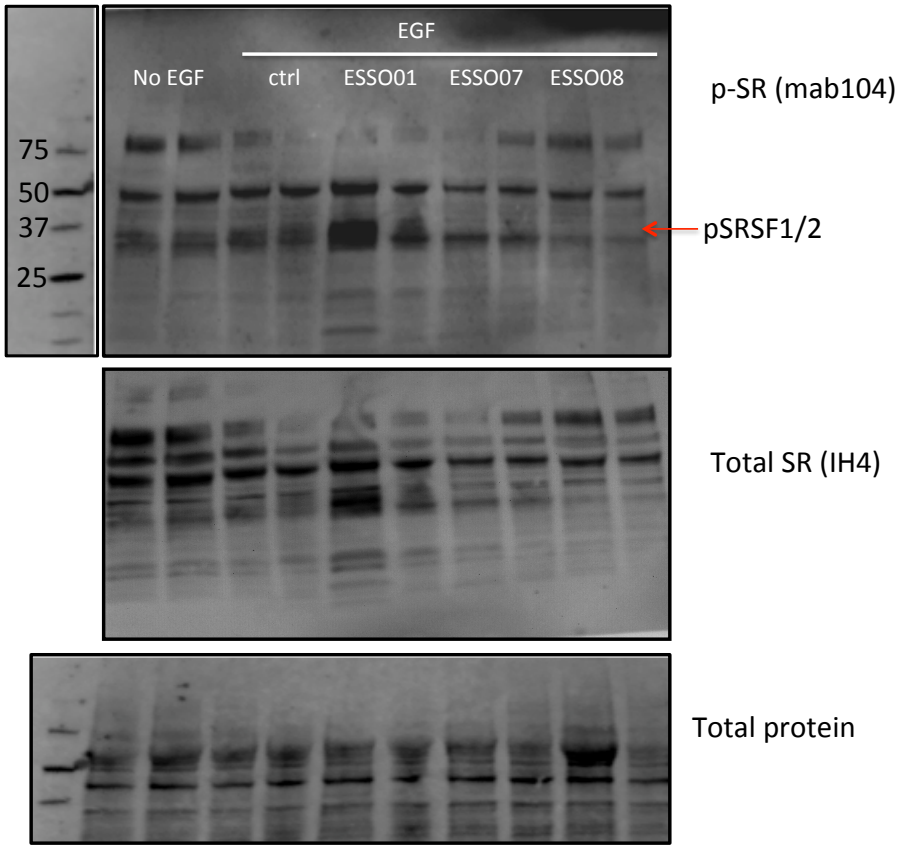


Figure 5-6 The effect of ESSO compounds on SR phosphorylation
PC3 cells were pre-treated with each ESSO compound for 1 hour prior to stimulation with 100ng/ml EGF. After 2 hour, proteins were extracted and SR protein expression and phosphorylation analysed by western blot.

5.2.4 Tumour growth is reduced by the anti-angiogenic compound, RepSox

Angiogenesis is induced by cancers in order to create their own blood supply and to allow further growth and metastasis. I have shown that ESSO07 (RepSox) increases anti-angiogenic VEGF isoforms through increased use of a distal splice site in *VEGF* exon 8 and demonstrated anti-angiogenic activity in two *in vitro* angiogenesis assays. It was next explored as to whether the anti-angiogenic activity of RepSox can be used to inhibit tumour growth. PC3 cells were pre-treated with RepSox at 10µM in culture for 48 hours before they were harvested and injected subcutaneously into nude mice. The PC3s were mixed with Matrigel prior to injection to aide their formation into tumours. 2 million cells were injected into each mouse. Two weeks post-injection, the tumours were excised and weighed (Figure 5-7). The tumours formed from PC3 cells that had been treated with RepSox had significantly reduced weights compared to control tumours.

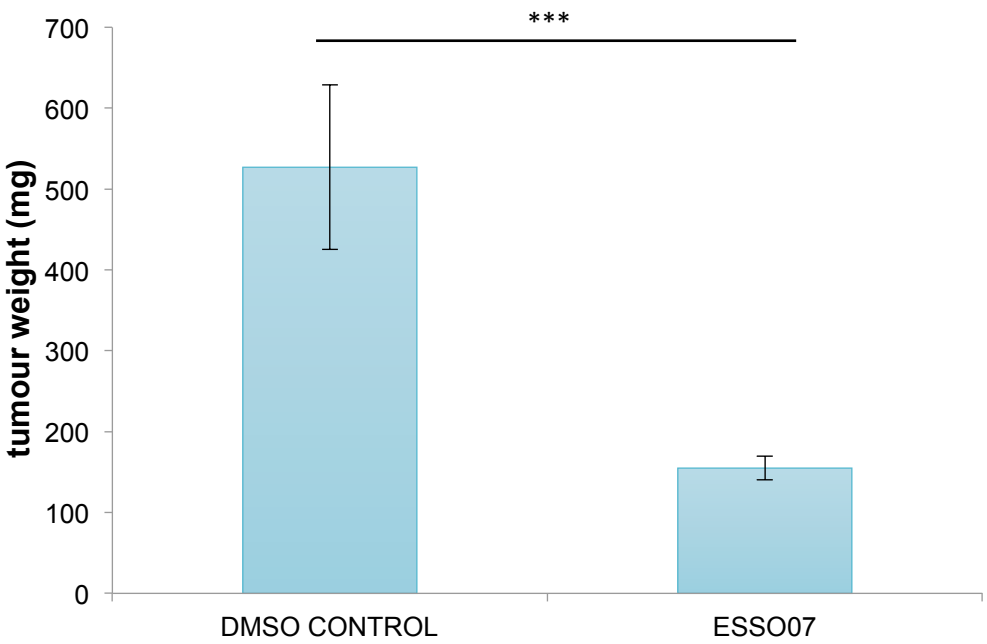


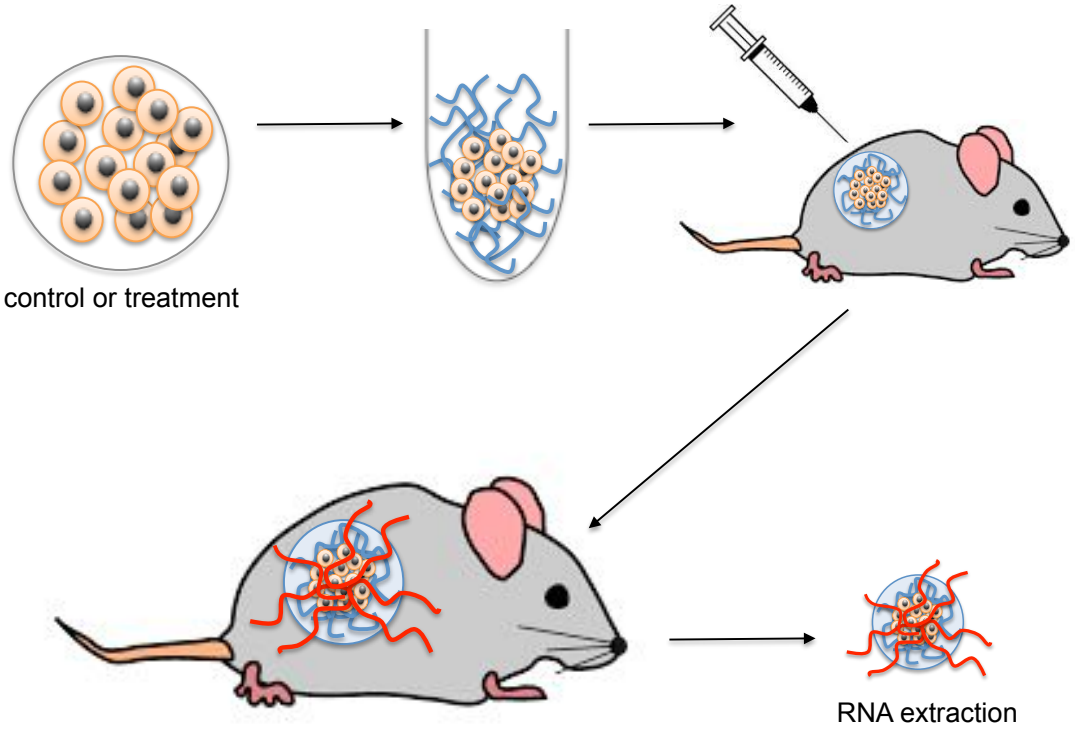
Figure 5-7 Pre-treatment of PC3 cells with RepSox leads to reduced tumour growth
*PC3 cells were cultured in medium containing 10µM RepSox (ESSO07) or DMSO for 48 hours. 2x10^6 PC3 cells were mixed with 400µl Matrigel and 10 µM RepSox. PC3 cells were injected subcutaneously into nude mice. After 14 days, the tumours were harvested and weighed. Control and treatment tumour weights were statistically compared using an unpaired t-test, p=0.0002***. Control n=6, ESSO07 n=11.*

5.2.5 In vivo neovascularization of Matrigel plugs is reduced by AEBSF

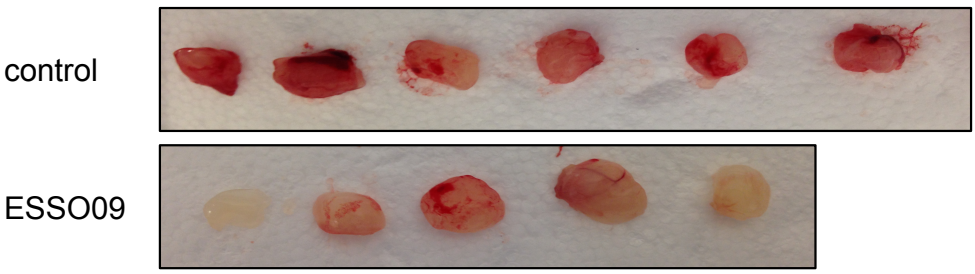
ESSO09 (AEBSF) is a sylfonyl fluoride serine protease inhibitor. Examples of serine proteases that are inhibited by this small molecule are chymotrypsin, thrombin and plasmin. There are reports that AEBSF also acts on targets other than serine proteases including NADPH oxidase (Abid et al., 2000). AEBSF significantly inhibited endothelial tube formation in an *in vitro* Matrigel angiogenesis assay so was consequently used to test for anti-angiogenic activity *in vivo*. For this purpose I used a Matrigel plug assay. PC3 cells were cultured in 10µM AEBSF for 48 hours. 2 million control or treated cells were then mixed with Matrigel and 10 µM AEBSF. Cells and Matrigel were kept on ice at all times prior to subcutaneous injection into nude mice. At 37 °C, Matrigel polymerizes to form a 3-dimensional plug of extracellular matrix proteins. The PC3 cells injected with

the plug produce angiogenic signals stimulating sprouting angiogenesis and infiltration of blood vessels into the Matrigel (Figure 5-8A). Four days after injection, the plugs were removed to assess neovascularization. Images were captured of the extracted plugs and Photoshop used to quantify the colour intensity of the plugs (Figure 5-8B). Matrigel plugs with no infiltrating vessels are colourless whereas neovascularization causes the plugs to become dark and bloody. Plugs that had been injected along with PC3 cells that had been pre-treated with AEBSF had significantly reduced colour intensity compared to control plugs (Figure 5-8C). RNA was extracted from each plug and qRT-PCR used to measure mouse *Vegfr-2* expression. VEGFR2 is highly expressed on endothelial cells, therefore, mouse-specific *Vegfr-2* primers were used to detect vasculature in the plugs. The comparative Ct method was used to quantify mouse *Vegfr-2* transcript levels with human GAPDH used as the reference gene. Angiogenesis was inhibited in plugs containing PC3 cells that had bee treated with AEBSF (Figure 5-8D).

A



B



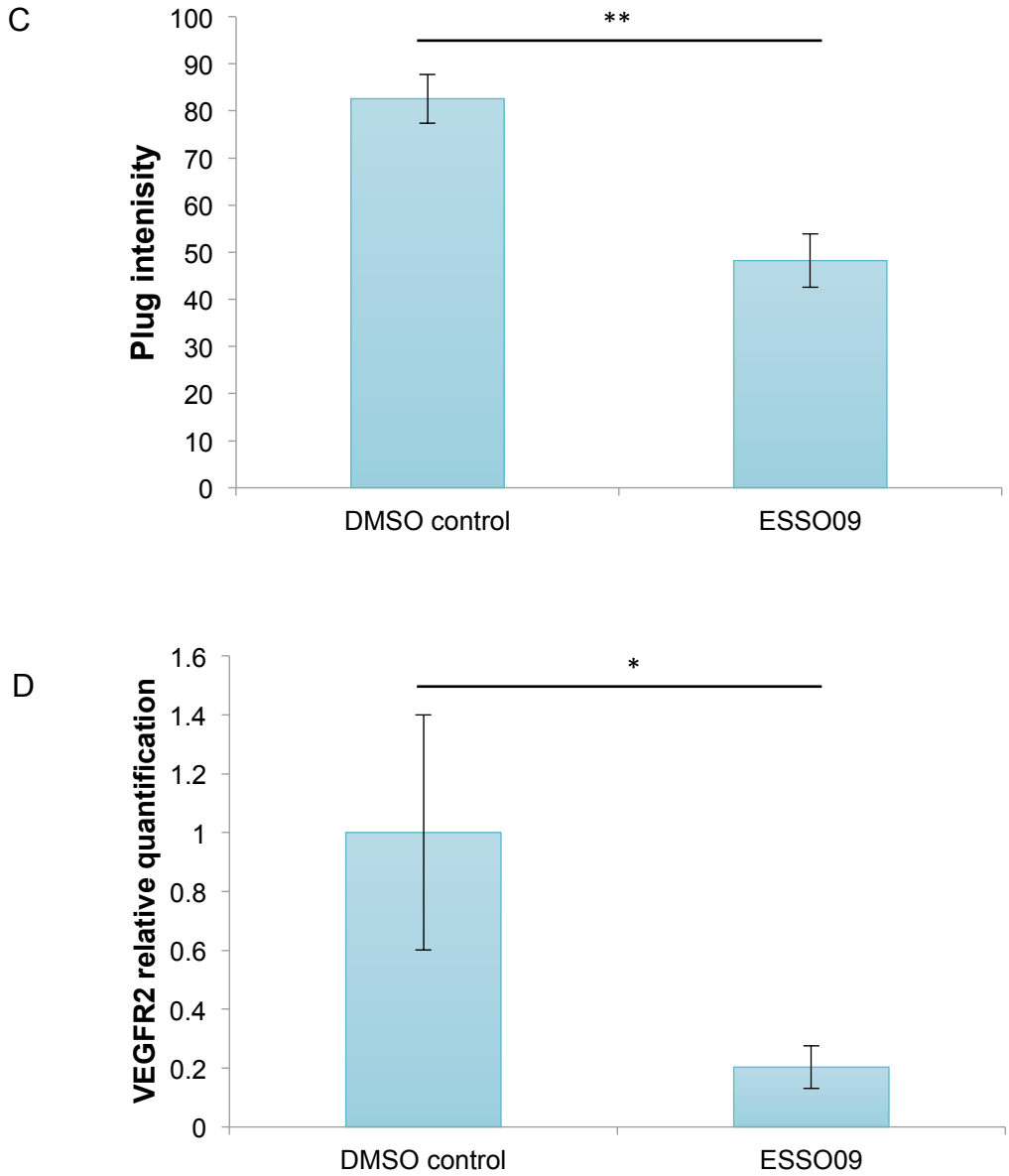


Figure 5-8 AEBSF reduces neovascularisation of Matrigel plugs in vivo
(A) PC3 cells were cultured for 48 hours in either 10µM AEBSF (ESSO09) or DMSO only. 2x10^6 PC3 cells were mixed with Matrigel and 10 µM AEBSF. The Matrigel and cells were injected subcutaneously into nude mice. Four days post-injection the plugs were removed and RNA was extracted. (B) Photographs were taken of each plug and (C) colour intensity quantified using ImageJ software. Control and treatment plugs were compared using an unpaired t-test, p=0.0002, control n=13, ESSO09 n=11. (D) RNA was extracted from the plugs to quantify VEGFR2 expression. Mouse VEGFR2 expression was normalised to human GAPDH. Relative VEGF expression was compared using a Mann-Whitney U test, p=0.0159, n=5.

A second, similar experiment was performed using another of the compounds, which was shown to reduce angiogenesis during endothelial-fibroblast co-culture. ESSO01 (trovafloxacin mesylate) did not reduce tubule formation during Matrigel angiogenesis assays but did show an inhibitory effect on endothelial cells in co-culture with fibroblasts and induced a shift from *VEGF* proximal to distal splice site selection in PC3 cells. Changes in VEGF isoform expression at the protein level following treatment with trovafloxacin mesylate was undetectable due to low total protein levels possibly because of a negative effect on PC3 cell growth. During endothelial-fibroblast co-culture, the cells were incubated in conditioned media taken from PC3 cells that had been treated with trovafloxacin mesylate. This suggests that trovafloxacin may not alter *VEGF* alternative splicing in endothelial cells or the secreted VEGF levels are too low for a change in splicing to have an anti-angiogenic effect. However, conditioned media taken from PC3 cells treated with trovafloxacin mesylate did reduce tubule formation during endothelial-fibroblast co-culture so may have activity in PC3 but not endothelial cell. Therefore, the compound did not affect endothelial cells when treated directly but reduced tube length after PC3 conditioned media was applied. To establish if a similar result is produced *in vivo*, PC3 cells were cultured in three varying concentrations of trovafloxacin mesylate. Matrigel was mixed with the treated or control PC3 cells before subcutaneous injection into nude mice. When the plugs were removed, after four days, colour intensity was quantified as previously described (Figure 5-9). Statistically, there was no difference between the control plugs and any of the treatment concentrations.

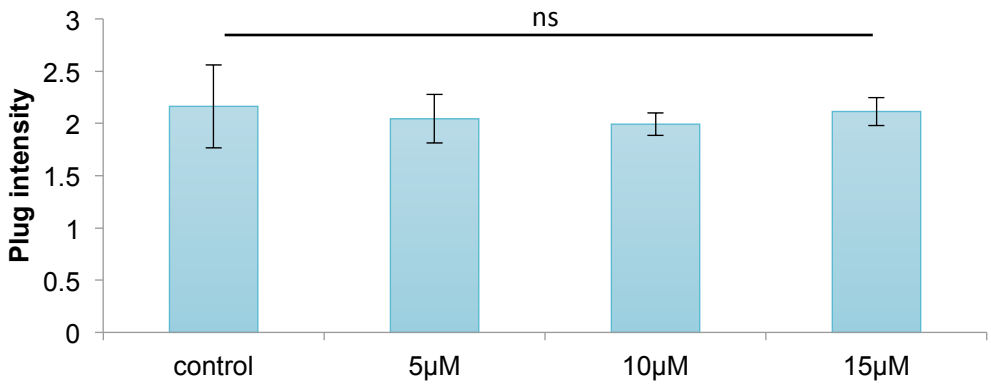


Figure 5-9 Trovafloxacin mesylate does not reduce neovascularisation in vivo PC3 cells were cultured for 48 hours in 5μM, 10μM, 15μM trovafloxacin mesylate (ESSO07) or DMSO only. 2x10^6 PC3 cells were mixed with Matrigel and 10μM trovafloxacin mesylate. The Matrigel and cells were injected subcutaneously into nude mice. Four days post-injection the plugs were extracted. Photographs were taken of each plug and colour intensity quantified using ImageJ software. One-way ANOVA was used to compared the treated plug to DMSO control with n=4 in each group. ns= no significant difference.

5.3 DISCUSSION

Tumours exploit angiogenesis in order to create their own blood supply for the delivery of oxygen and nutrients. The angiogenic switch is a rate-limiting step within tumourigenesis. Solid tumours only reach a volume of around $1mm^3$ without stimulating angiogenesis or co-opting pre-existing blood vessels (Bergers and Benjamin, 2003). VEGF is established at the most important regulator of angiogenesis in both normal physiology and during tumour growth. The alternative splicing of *VEGF* pre-mRNA creates many protein isoforms including a sub-family with anti-angiogenic properties, $VEGF_{xxx}b$ proteins. A splicing-sensitive fluorescent reporter was designed to mimic the alternative splicing event that creates the pro-/anti-angiogenic VEGF isoforms. The reporter was used in a screen to identify small molecules that can alter splice site selection resulting in less $VEGF_{xxx}$ protein and more $VEGF_{xxx}b$. Nine compounds were identified as able to change the ratio of reporter splice site selection. I aimed to assess if these small molecules could reduce angiogenesis by increasing the expression of anti-angiogenic VEGF isoforms. This was achieved using a series of assays to measure anti-angiogenic activity of the molecules, both *in vitro* and *in vivo*.

5.3.1 The use and limitations of angiogenesis assays

In a healthy individual, endothelial cells are not proliferative but remain quiescent awaiting angiogenic stimulation. In culture, endothelial cells adapt and become proliferative allowing them to be utilised for *in vitro* migration, proliferation and differentiation assays. Endothelial cells acquire proliferative, angiogenic character in culture, which can potentially cause difficulty when assessing if a factor or molecule is pro-angiogenic. Care must be taken when using primary endothelial cells as they begin to lose their endothelial phenotype and expression of markers after multiple passages. HUVECs are very commonly used for such assays however they are derived from umbilical vein when angiogenesis is predominantly a process of the microvasculature. HMVECs may be a more suitable cell type for use *in vitro*.

In vitro angiogenesis assays that use only endothelial cells are quick, easily quantified and usually reproducible. But, they are not able to reflect the complex interactions that occur physiologically i.e the effect of flow and shear stress on microvessels or indirect effects on endothelium via factors produced by other cell types, which then modulate endothelial cells. To assess pro- or anti-angiogenic activity of a molecule, it is common practice to use multiple *in vitro* assays and then *in vivo* models.

Tubule formation assays are widely used for measuring the effect of molecules and drugs on angiogenesis. These assays are thought to simulate the later phases of angiogenesis, as tubules develop and lumen forms. Tubules form when endothelial cells are seeded on a gel matrix of basement membrane and extracellular proteins. Which matrix is used in tubule formation assays is an important consideration. Endothelial cells proliferate on matrices of collagen I and III but do not form tubules well, whereas, on collagens IV and V the cells do not proliferate much but do develop into tubes. I used Matrigel during angiogenesis assays, this is the most commonly used and potent gel matrix for use in such assays. There is dispute whether the endothelial tubules formed accurately simulate microvessels. The cells do associate with each other using tight junctions but whether they form lumen is debated (Bikfalvi et al., 1991; Connolly et al., 2002; Grant et al., 1991). It has also been observed that other cell types, besides endothelial cells, can form tubule-like structures on Matrigel, including fibroblasts and some cancer cell lines (Donovan et al., 2001).

The *in vitro* Matrigel assays described were performed in 2D with endothelial cells plated on a thin layer of the gel matrix. Similar assays can also be performed in 3D to more accurately simulate angiogenesis *in vivo*. Such 3D assays often involve endothelial cells placed between two layers of matrix. Within around two weeks in culture, endothelial cells will form a 3D tubule complex (Gagnon et al., 2002). However, 3D angiogenesis assays are more challenging to perform and quantify, the matrix must be optimised for appropriate thickness to allow oxygen to reach the cells. The enhancement or inhibition of angiogenesis can be

measured by fixing, sectioning and staining the gels for microvessels. 3D tubule formation assays are more time consuming in regards to experimental procedures and quantification.

One method to mimic angiogenesis *in vitro* is co-culturing stromal cells along with endothelial cells. Cells commonly used include fibroblasts or smooth muscle cells. An extracellular matrix can be externally provided in co-cultures, but when fibroblasts are used this is not required as the fibroblasts naturally produce the necessary extracellular proteins. The tubules formed in this assay do possess lumen and the tubule networks more comparable to *in vivo* capillaries than endothelial tubes formed on Matrigel alone. However, the assay has a much longer time course (14 days) compared to simple endothelial-only Matrigel assays and the matrix proteins produced by fibroblasts may alter making the assay more variable (Donovan et al., 2001). The major advantage of co-culture is that an exogenous matrix in not required due to natural secretion from fibroblasts, but due to the total length of the assay effects to tubule formation may be caused by changes in endothelial cell proliferation.

Compounds that demonstrate potent activity *in vitro* will not necessarily have the same effect *in vivo*. Therefore, potential anti-angiogenic molecules must also be evaluated using animal models of angiogenesis. Matrigel plugs are a commonly used and simple method for measuring the effect of a test substance on the angiogenic response *in vivo*. Matrigel is useful for *in vivo* applications. The matrix is in liquid form below 4°C but solidifies following injection. Following, removal of the plug from a mouse after several days, neovascularisation can be quantified in various ways. I used colour intensity as a measurement of haemoglobin presence and extracted RNA to quantify an endothelial cell marker. Plugs can also be sectioned and stained to visualise vessel formation within the plug.

Angiogenesis is a one of the hallmarks of cancer and the angiogenic switch a rate-limiting step. This leads to many potential anti-angiogenic molecules to be

tested for anti-tumour activity. In my investigations, I used a common approach of subcutaneous injection of cancer cells and measuring the subsequent tumour formation. This method allows measurement of tumour size throughout the experiment time course with tumours easily excised and weighed. Angiogenesis itself cannot be measured or visualised during the experiment, however, if the study was large enough, tumours may be excised from sub groups of mice at various time points to determined how a drug affects angiogenesis progression and at what stage the anti-angiogenic drug predominantly takes effect. If promising anti-tumour activity is shown in subcutaneous cancer models, drugs should be investigated further in orthotopic models, as the microenvironment of a tumour is a very important factor during tumour angiogenesis (Watnick, 2012). A significant drawback to many in vivo cancer models is that tumours form and can grow very quickly over a short period of time. In reality cancer development takes place over many months or even years. Further pre-clinical investigation of anti-angiogenic compounds is needed, possibly using transgenic spontaneous tumour models, which overexpress specific oncogenes to promote tumour development over time (Di Carlo et al., 1999; Hanahan, 1985).

Several angiogenesis inhibitors have shown promising anti-angiogenic effects during pre-clinical investigation *in vitro* and *in vivo*, with some causing reduced tumour growth and development in mouse models. Despite this, many have gone on to produce disappointing results during clinical trials. Angiogenesis assays using isolated endothelial cells *in vitro* are a useful tool for early investigation and screening of potential angiogenesis inhibitors due to their speed and reproducibility, but must be followed with several *in vivo* angiogenesis models. The *in vivo* angiogenesis experiments I used involved pre-treating cultured cells before injection. If the compounds discussed here were to be tested further for use as anti-angiogenic drugs they would ideally be administered to the animal systemically and to measure their effect on established tumours.

5.3.2 In vitro *assays identified compounds with potential anti-angiogenic activity*

VEGF is highly expressed by various cell lines including cancer cells. Angiogenesis is stimulated by secreted VEGF binding to VEGFRs on the endothelium inducing branching and vessel growth. The lead compounds identified were initially tested for anti-angiogenic activity by treating HUVECs for 48 hours with 10µM of each small molecule before harvesting the cells and allowing them to form tubules on Matrigel matrix of basement membrane proteins. Two of the small molecules, GW2974 and AEBSF, significantly reduced the length of endothelial tubules formed and the number of branch points of the tubes 5 hours after seeding (Figure 5-1). This indicates these compounds can produce a direct effect on endothelial cells diminishing their tube forming ability, whereas *in vivo*, angiogenesis is usually stimulated by VEGF in a paracrine manner via secretion from surrounding cells or tumour.

The lead compounds were identified for their ability to alter *VEGF* alternative splicing using a reporter construct overexpressed in PC3 cells. There has been limited investigation into endothelial VEGF expression and the role of autocrine VEGF signalling on endothelial cells. It has been established that expression of VEGF from endothelium is low but involved in maintaining vessel survival. Vascular development is normal in transgenic mice with *Vegf* knocked out in only endothelial cells. However, some vascular beds progressively degenerate with around half of knock-out mice experiencing sudden death within 25 weeks after birth (Lee et al., 2007). *In vitro*, knock-down of *VEGF* using siRNA induces cell death in HUVECs. HUVECs express low levels of VEGF compared to many other cell types and a significant proportion of the protein is not secreted from the cell. Endothelial derived VEGF is thought to be involved in upregulating survival genes including cell cycle and mitochondrial genes and required for homeostasis in developed vessels (Domigan et al., 2015). Alternative splicing of *VEGF* isoforms within the endothelium itself is poorly understood. The two lead compounds that reduced tube formation on Matrigel were GW2974, an inhibitor of Erb1/2 receptor tyrosine kinases and AEBSF, a non-specific serine protease inhibitor.

Neither of the compounds has been previously linked with alternative splicing events in endothelial cells or other cell types. The screen in which the splicing modifiers were identified was performed using PC3 cells, not endothelial cells. It is possible that the effect on tube formation seen by directly treating endothelial cells is also mediated my mechanisms independent of VEGF alternative splicing.

Pro-angiogenic VEGF is known to be upregulated in prostate cancers tissues and in prostate cancer cell lines. VEGF expression is higher in more aggressive cell lines from prostate cancer (Harper et al., 1996). Inhibiting angiogenesis through manipulation of $VEGF_{xxx}$/$VEGF_{xxxb}$ alternative splicing can reduce tumour growth in models of prostate cancer and others (Amin et al., 2011; Gammons et al., 2014; Mavrou et al., 2014). As the chemical library screen that isolated the list of lead compounds was performed using PC3 cells and tumour angiogenesis is induced through pro-angiogenic VEGF secretion from tumour cells, PC3s were used again as part of additional angiogenesis assays. HUVECs were cultured on Matrigel with conditioned media taken from PC3 cells that had been treated with each of the lead compounds (Figure 5-). If the compounds were altering splice site selection of *VEGF* pre-mRNA and increasing $VEGF_{xxx}$b isoform expression, this will be present in the media and influence HUVEC tube forming ability. Tube formation was significantly reduced by conditioned media from eight out of nine compound treatments. The media was taken directly from cultured PC3 cells and not concentrated. Therefore the media will contain secreted proteins such as $VEGF_{xxx}$b but also remaining concentrations of the small molecule inhibitors. The effect seen on HUVECs may be a combined effect of the compound directly and changes in VEGF isoform expression and any other secreted proteins induced or inhibited by the compound treatment in PC3 cells.

In addition to using Matrigel angiogenesis assays, the effect of the lead compounds in vitro was further investigated using a second angiogenesis assay, endothelial-fibroblast co-culture (Figure 5-). During the co-culture, endothelial cells are given a longer time in which to associate and form vessel-like structures compared to the short Matrigel angiogenesis assay. HUVECs were seeded into

wells with confluent fibroblasts and cultured without stimulation for 48 hours before adding compound treated PC3 conditioned media for a further 48 hours. In this case two out of the nine conditioned media samples caused a significant reduction in endothelial vessel growth, trovafloxacin mesylate (ESSO01) and RepSox (ESSO07). Again, this effect may be produced by $VEGF_{xxx}b$ expression, remaining compound and/or other secreted proteins.

In an additional experiment, GW2974-treated PC3 conditioned media reduced endothelial tube formation but the effect was rescued by the addition of a $VEGF_{xxx}b$-specific neutralising antibody (Figure 5-). This indicates that anti-angiogenic VEGF isoforms are indeed involved in the inhibitory effect of GW2974 on endothelial tube formation. To further investigate how the lead compounds mediate their anti-angiogenic activity and their influence on *VEGF* alternative splicing, the effect on SR protein phosphorylation was analysed. SR proteins have been demonstrated to be important in determining *VEGF* proximal and distal splice site selection and it is established that their activity is modulated via phosphorylation. RepSox and GW2974 treatment reduced the phosphorylation of some SR protein band on western blot analysis. The antibody used to measure phosphorylation detects all members of the SR protein family, therefore, which SR proteins are effected by the compounds cannot be fully determined. Specific antibodies or immunoprecipitation should be used in the future for further investigation into the mechanism of action of both molecules.

Novel inhibitors of angiogenesis and tumour growth

I wanted to further investigate the several lead compounds that showed functional activity during *in vitro* assays and increased $VEGF_{xxx}b$ mRNA/protein. This included trovafloxacin mesylate (ESSO01), RepSox (ESSO07) and AEBSF (ESSO09). A simple Matrigel plug assay was used to demonstrate inhibition of neovascularisation by the anti-angiogenic compounds *in vivo*. The composition of Matrigel is similar to basement membrane proteins that associate with microvessels. Matrigel also contains various growth factors associated with the matrix that stimulate vessel sprouting.

PC3 cells that had been pre-treated for 48 hours with the lead compounds, trovafloxacin mesylate (Figure 5-) or AEBSF Figure 5-), were mixed with Matrigel to examine inhibitory action of the molecules. Four days after subcutaneous injection of nude mice, the plugs were harvested and imaged. Matrigel is colourless making larger infiltrating vessels clear to see by eye. More vessel growth made the plugs darker and bloody. Quantification of the plug colour was used as a simple method of measuring neovascularisation. AEBSF treatment of PC3 cells caused a reduction in the colour intensity of the harvested plugs indicating the treatment reduced the ability of PC3s to induce angiogenesis. RNA was extracted from each plug and qRT-PCR performed to measure expression of mouse *Vegfr-2* mRNA as a marker for the presence of endothelial cells. Human GAPDH expression was used as a reference gene for normalisation as it is likely that the majority of the extracted RNA is from PC3 cells. AEBSF treatment reduced the induction of neovascularisation by PC3 cells. AEBSF is a non-specific serine protease inhibitor. The compound has not previously been linked with angiogenesis or alternative splicing. AEBSF inhibits various serine proteases such as kallikrein and urinary plasminogen activator (uPA). The drug has previously been tested in models of embryo implantation in rats and to have an inhibitory effect on cell adhesion and protein secretion *in vitro* (Jiang et al., 2011). The inhibitor also has other activities of which the exact mechanism of action is unclear. For example, expression of the transcription factor, CHOP (C/EBP homologous protein), is induced by PI3K activation but this induction is blocked by AEBSF. Additionally, AEBSF inhibits NADPH oxidase. NADPH oxidase produces reactive oxygen species and is important for the proliferation and migration of endothelial cells (Abid et al., 2000).

Trovafloxacin mesylate reduced tube formation in endothelial-fibroblast co-culture *in vitro* and increased *VEGF* distal splice site selection, as shown by RT-PCR, but did not cause a change to Matrigel plug neovascularisation at any of the concentrations tested (Figure 5-). The drug is a broad spectrum fluoroquinolone antibiotic agent which acts by inhibiting bacterial DNA gyrase and topoisomerase IV. Trovafloxacin was previously used as an antibiotic but removed from use in

patients due to an association with liver damage (Lazarczyk et al., 2001). Other fluoroquinolones within the same class have demonstrated some anti-angiogenic and anti-tumour activity alone and when combined with chemotherapy (Kamat et al., 1999).

The compound RepSox reduced the formation of endothelial tubules during co-culture of fibroblasts and HUVECs (Figure 5-). Tumour formation from subcutaneous PC3 xenografts was significantly reduced by treatment of the PC3 cells with RepSox. RepSox is a specific inhibitor of ALK5, a type-I TGF-β1 receptor. There has been some investigation into TGF-β1 regulation of VEGF expression including alternative splicing of the pro and anti-angiogenic isoforms. Podocytes treated with TGF-β1 displayed increased anti-angiogenic $VEGF_{xxx}b$ transcripts and reduced pro-angiogenic $VEGF_{xxx}$ (Nowak et al., 2008), whereas I have shown inhibiting the TGF-β1 receptor, ALK5, with RepSox is anti-angiogenic. (Gammons et al., 2013a; Gammons et al., 2014; Gammons et al., 2013b)

The role of TGF-β1 signalling in angiogenesis and cancer is extremely complex and context dependent. TGF-β1 is implicated as a tumour suppressor but also pro-metastatic (Papageorgis and Stylianopoulos, 2015). Additionally, the growth factor is reported by some to promote angiogenesis in tumours but also inhibit angiogenesis in other studies (Pardali and Moustakas, 2007). Mice with gene deletion of TGF-β1, TβRII and ALK5 have vasculogenic and angiogenic abnormalities. The chick chorioallantoic membrane angiogenesis assay showed TGF-β1 to be capable of strongly inducing an angiogenic response (Yang and Moses, 1990). Whereas *in vitro*, TGF-β1 reduced proliferation and migration of endothelial cells and stimulates apoptosis (Pollman et al., 1999). It has therefore been proposed that TGF-β1 stimulates angiogenesis *in vivo* via an indirect mechanism causing induction of VEGF or other pro-angiogenic stimuli (Pardali and Moustakas, 2007). TGF-β1 and VEGF are often both expressed in vascularising tissues and many tumour types. CHO cells expressing TGF-β1 form large, highly vascular tumours when subcutaneously injected into mice. TGF-β1 neutralising antibodies can reduce the growth of these tumours (Ueki et al.,

1992). In prostate cancers, increased TGF-β1 expression is associated with greater angiogenesis. Use of TGF-β1 antibodies reduced blood vessel density and tumour growth in mouse models (Tuxhorn et al., 2002). In contrast, TGF-β1 is highly expressed by tumours of the gallbladder, inhibiting their growth and angiogenesis. Other models suggest, TGF-β1 can reduce VEGF expression in pancreatic cancer cells (Pardali and Moustakas, 2007).

Chapter 6

Discussion and future direction

6.1 Alternative splicing dysregulation in disease and the potential for therapeutic intervention

mRNA splicing is a crucial process for functional cell physiology. Alternative splicing is an important mechanism of post-transcriptional gene regulation. Aberrant splicing and inappropriate expression of certain splice isoforms can lead to various diseases, including cancers. Attempts have been made in pre-clinical studies to target drugs to modify dysregulated splicing patterns as a potential method to treat disease. Various methods have been employed including using oligo nucleotide to base pair with pre mRNA to promote or inhibit the use of specific splice sites (Crooke, 2004). Similarly, exon specific RNA interference has been used to downregulate specific splice isoforms (Celotto and Graveley, 2002).

Additionally, chemicals can be used to change splicing patterns by directly associating with transcripts or modulating the action of splice factors and their upstream regulators. Examples include amiloride, a diuretic that was demonstrated to increase the expression of pro-apoptotic splice isoforms of certain genes in leukaemia cells (Chang et al., 2011). Drugs have also been used to target the alternative splicing of *SMN2* transcripts to increase the expression of functional protein isoforms and potentially treat spinal muscular atrophy (Naryshkin et al., 2014; Palacino et al., 2015).

It also clearly established that the regulation of alternative splicing is highly distorted during cancer and several splicing factors are confirmed oncogenes that contribute to cancer development and progression. One of the genes heavily investigated regarding association with cancer, and the effect of alternative splicing, is *VEGF*. Traditionally thought of as a potent angiogenic growth factor, in 2002, Bates *et al* detected a family of alternatively spliced *VEGF*

isoforms with very different properties to the canonical proteins. $VEGF_{xxx}b$ isoforms differed from the pro-angiogenic family by only six amino acids at the C terminal of the protein isoforms. The $VEGF_{xxx}b$ isoforms have been demonstrated to be anti-angiogenic, reduced vessel permeability and reduce tumour growth compared with the $VEGF_{xxx}$ isoforms. The anti-angiogenic isoforms are highly expressed in many normal tissues but their expression is reduced in tumours and highly angiogenic tissues (Bates and Harper, 2005). As such, numerous studies have focused on understanding the mechanisms that regulate *VEGF* alternative splicing and if these mechanisms are disrupted during disease.

SR proteins are trans-acting regulators of alternative splicing. Binding of SR proteins to specific sequences within an mRNA transcript can promote the use of a splice site. SR proteins are often activated via phosphorylation by kinases, which in turn are often regulated by other kinases upstream (Giannakouros et al., 2011). SR proteins have been demonstrated to be important in the selection of the splice sites that produce pro- or anti-angiogenic VEGF. SRSF1 binding promotes the use of a proximal splice site in *VEGF* exon 8 leading to $VEGF_{xxx}$ expression. Inhibition of the SRSF1-activating kinase, SRPK1, reduces expression of $VEGF_{xxx}$ and increases used of a distal splice site creating anti-angiogenic $VEGF_{xxx}b$. As a kinase enzyme, SRPK1 can be targeted with small molecule inhibitors. Via changes in VEGF splice isoform expression, SRPK1 inhibitors, such as SRPIN340, can reduce tumour growth (Amin et al., 2011; Oltean et al., 2012).

6.2 Using splicing-sensitive fluorescent reporters as a screening tool

As changing the expression of VEGF alternatively spliced isoforms with SRPK1 inhibitors was able to reduce tumour growth *in vivo* (Amin et al., 2011; Mavrou et al., 2014), further investigation was required to find additional small molecules that may target *VEGF* alternative splicing. Elucidated compounds have the potential to be used for disease treatment and further our understanding how *VEGF* exon 8 splicing is regulated. The method used for this purpose was a splicing-sensitive fluorescent reporter designed to mimic the alternative splicing pattern which creates either pro- or anti-angiogenic VEGF isoforms. Initial validation of the VEGF SSFR construct required transfection of the reporter plasmid into several cell types and monitoring the pattern of reporter alternative splicing when cells were treated with SRPK1 inhibitors. This validation and preliminary screens suggested the VEGF SSFR may be a useful tool for finding molecules that can influence *VEGF* splicing. Previous studies have used SSFRs to screen small molecule or siRNA libraries (Newman et al., 2006; Stoilov et al., 2008).

High-throughput and smaller screens usually involve primary and secondary stages followed by further validation in functional assays (Macarron et al., 2011; Pereira and Williams, 2007). The primary screen was performed using the PC3 prostate cancer cell line. A fluorescent plate reader was used to measure the levels of EGFP and dsRED in cells treated with a library of 1,280 small molecules. The compounds were part of a repurposing library i.e. all the compounds had been previously FDA-approved for a different use. As VEGF is a key growth factor during tumourigenesis and anti-angiogenic therapy has the potential to inhibit the growth of many cancers, I aimed to find molecules that could change *VEGF* splicing to increase the expression of anti-angiogenic VEGF and reduce pro-angiogenic isoforms. Molecules that increased reporter distal splice site or reduced proximal splice site selection, measured by fluorescent protein expression, were used in the secondary screen that measured EGFP and dsRED

using flow cytometry. Nine compounds were validated in the secondary screen and used in functional assays.

The screen performed was relatively small scale compared with drug discovery undertaken by pharmaceutical companies. There are advantages and drawbacks to every screening method. Large compounds libraries often contain many drug candidates which may not be active, not be able to permeate the cell membrane or may be toxic when used *in vitro* or *in vivo*. Advantages of the VEGF SSFR screen include that the compounds of the repurposing library have known cellular targets and the screen was performed in live cells rather than using cell extracts or isolated proteins.

A fluorescent plate reader was utilised during the primary screen to quantify dsRED and EGFP levels in cells treated with the compound library, as a measure of reporter proximal or distal splicing. In analysis of the primary screen, over 300 compounds were significantly increased EGFP or decreased dsRED. This number was reduced to 9 following the secondary, flow cytometric screen. This indicated that the fluorescent plate reader produced many 'hits' that were not validated. Other methods of fluorescent measurement may improve the screening accuracy such as high-content cell analysis (e.g using IN Cell analyser) or high-throughput flow cytometry.

6.3 Investigating the anti-angiogenic activity of small molecules *in vitro* and *in vivo*

The aim of screen for molecules that can modify *VEGF* splicing was to ultimately use such molecules to inhibit angiogenesis *in vivo*. Before animal studies are performed, there are many *in vitro* angiogenesis assays that can be used (Auerbach et al., 2003; Donovan et al., 2001). Each angiogenesis assay has advantages and limitations. Endothelial tube formation assays are simple to perform and reproduce but do not represent angiogenesis as accurately as some other assays. Co-culture of endothelial cells with stromal cells is more physiological in vitro angiogenesis assay but still does not mimic the complexity of angiogenesis in the tumour microenvironment. Similarly, there are numerous animal models of angiogenesis that are commonly used to test potential anti- or pro-angiogenic molecules, including the chick chorioallantoic membrane angiogenesis assay, the corneal pocket assay and a Matrigel plug assay. Additional tumour models are required to investigate if anti-angiogenic activity of a novel compound inhibits tumour growth (Staton et al., 2009).

Several of the lead compounds identified during the VEGF SSFR screen exhibited anti-angiogenic activity *in vitro* or *in vivo*. Some also increased *VEGF* distal splice site selection and increased $VEGF_{xxx}b$ the protein level. It is not clearly established if the anti-angiogenic effects observed are predominantly mediated by changes in the ratio of VEGF splice isoforms. While I have strong evidence that 165b is involved, I cannot rule out completely other additional anti-angiogenic mechanisms. A rescue experiment was performed using a $VEGF_{xxx}b$-specific neutralising antibody during a Matrigel angiogenesis assay. Addition of the neutralising antibody partially rescued the anti-angiogenic effect promoted by GW2974 EGFR inhibitor. Whether *VEGF* alternative splicing is the major mechanism that mediates the respective *in vivo* anti-angiogenic and anti-tumour activity of AEBSF and RepSox requires further investigation.

Anti-angiogenesis as a cancer treatment has had relatively limited success. This has been attributed to several mechanisms, including resistance to therapy and

reduced delivery of chemotherapeutics to the tumour (Carmeliet and Jain, 2011; Kerbel and Folkman, 2002). Small molecules that reduce the expression of $VEGF_{xxx}$ while promoting the endogenous anti-angiogenic factor, $VEGF_{xxx}b$, is an intriguing strategy for cancer therapy.

In conclusion, the molecules identified to modulate VEGF splicing and inhibit angiogenesis require further investigation to clearly define their mechanism of action and explore their potential as anti-angiogenic/anti-tumour therapeutics. This will require detailed molecular analysis to elucidate the pathways that mediate the effects on VEGF alternative splicing and to establish which other factors are involved in the anti-angiogenic activity. Several additional *in vivo* models should be used to further demonstrate the angiogenic activity of the hit compounds, to determine optimal dosing of the compounds and to establish if they provide functional effects on angiogenesis when administered systemically.

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